Characterization of the cellular receptor for the hepatitis A virus

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CHARACTERIZATION OF THE CELLULAR RECEPTOR FOR THE HEPATITIS A VIRUS

submitted by Adam J. Collier

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CONTENTS

ABSTRACT

1. Hepatitis A virus (HAV) binds to a variety of cell types, and agglutinates red blood cells. Binding of the virus to a susceptible cell line, FRhK-4, is saturable and exhibits positive cooperativity, with a Hill coefficient of 2.10. The sedimentation rate of HAV on a sucrose gradient is increased by preincubation with solubilized FRhK-4 cells, indicating the isolation of a virus - receptor complex.

2. Two HAV - specific monoclonal antibodies inhibited haemagglutination, virus infection of FRhK-4 cells, and virus binding to FRhK-4 cells. A third HAV - specific monoclonal antibody failed to inhibit haemagglutination and virus infection of an FRhK-4 cell monolayer, but inhibited binding of virus to FRhK-4 cells. The two neutralizing monoclonal antibodies competed for the same site on the virus surface whilst the non - neutralizing monoclonal antibody did not.

3. The cellular receptor for HAV on FRhK-4 cells is trypsin sensitive, whilst agglutination of red blood cells can be inhibited by a fucose - specific lectin from Lotus tetragonolobus, or preincubation of the virus with a fucosylated glycoprotein, thyroglobulin. Binding of virus to FRhK-4 cells is not inhibited by a fucose - specific lectin.

1.INTRODUCTION

1.1 HEPATITIS A VIRUS (HAV)

1.1.1 History

The first recorded occurrences of hepatitis due to an infection with the Hepatitis A virus were in the 17th and 18th centuries, the earliest of which was in Minorca, Spain in 1745 (Hirsh, 1886). Reports of 'epidemic jaundice' however, go as far back as the writings of Hippocrates, and ancient China (Zuckerman, 1983). The disease has most commonly been associated with military campaigns, as a result of the fall in standards of hygiene, indeed the disease was so prevalent in the 18th and 19th centuries that it was dubbed 'Kreigsickterus' by the German troops and 'jaunisse des camps' (campaign jaundice) by the French.

Early clinicians assumed that the symptoms were due to hepatic obstruction until it began to be recognized to be as a result of an infectious agent. This view was advanced by Weil in 1886 when he described a severe form, known as infectious jaundice caused by leptospires, as opposed to the milder cases of epidemic catarrhal jaundice. It was MacDonald in 1908 who finally proposed that the disease was caused by a virus acting on a "previously damaged liver" (McDonald,1908). The epidemiological studies of Blumer in 1927 established the existence of an infection caused by what we now know as Hepatitis A virus, while studies conducted by scientists during World War II on human volunteers established separate HAV and HBV infections by attempting to cross - protect with convalescent sera (Findlay and Willcox, 1945; Oliphant et al, 1943).

In 1947 the terms, 'Hepatitis A' to describe epidemic jaundice and 'Hepatitis B' to describe homologous serum jaundice were introduced by MacCallum (1947), terms which have since been adopted by the World Health

Organization (W.H.O. 1973; 1977). Between the late 1950's and early 1970's seroepidemiological studies further defined the relationship between Hepatitis A and Hepatitis B infections, with the causative virus of hepatitis A finally being identified in 1973 (Feinstone et al). Since then sensitive immunoassays have been developed that can detect the presence of HAV, and in 1979 Provost and Hillman were successful in cultivating HAV in tissue culture, laying the foundations for more extensive studies of the virus.

1.1.2 Epidemiology

In many parts of the world HAV is still endemic, whilst recurrent epidemics are a feature of the developed and developing world. In the U.S.A there are approximately 22 reported cases of viral hepatitis per 100,000 total population; 44% of these are as a result of HAV infection, 45% HBV infection, 5% non-A, non-B infection (Hepatitis C virus, Hepatitis E virus) and 6% unknown (MMWR, 1988). These figures however, represent only a fraction of the total infected, the others remaining unreported as a result of relatively mild symptoms (Koff et al,1973,Marier,1977). In many countries HAV epidemics follow approximate seven - year cycles reflecting the ebb - and - flow of susceptibility within a population. In temperate regions HAV distribution also follows seasonal variations, with a higher incidence of infection during the Autumn and Winter months. The figures for this pattern however, are easily distorted by epidemics and the influx of tourists, but the reason for seasonal changes have yet to be explained. The age distribution of a population must also be noted. Studies have shown that where sanitation is poor and living conditions crowded, close to 100% of children are infected and therefore acquire life - long immunity, often with little or no symptoms (Ajdukiewicz et al,1979; Barin et al,1980; Frosner et al,1979; Gust et al,1979; Hilleman et al,1975; Maynard et al, 1976; Szmuness et al 1976; 1977). As a result, though the virus is prevalent in these countries, it represents a major health hazard only to those travelling in from elsewhere. However, in developed and

developing nations, as sanitation improves fewer children are being infected and do not acquire a natural immunity, and the population is therefore left unprotected against a challenge from the virus, and epidemics may occur. Several factors have been identified as posing a particular risk and require special emphasis. In the U.S.A. young children attending day - care centres often pass the virus from one to another, and subsequently carry it home to parents and older siblings (Hadler et al, 1980; Beneson et al, 1980) Sexual transmission amongst homosexual men also represents a problem, with an annual seroconversion rate of 22% noted in a study in Seattle, Washington (Corey and Holmes, 1980). In the developing and under - developed world transmission as a result of the consumption of food, particularly shellfish harvested from water contaminated with human faeces is an important cause, especially since the shellfish are able to concentrate enteroviruses by up to 60 - fold and are often eaten raw (Mitchell et al, 1966). It was thought that water - borne infections accounted for a proportion of infections but since the identification of the hepatitis E virus these figures have to be treated with extreme caution (Khuroo, 1980). HAV is rarely transmitted by blood - to - blood contact, in fact the prevalence of anti - HAV antibodies in patients with a history of multiple transfusions is no higher than for others of a similar socioeconomic background without such exposure (Papavangelou et al, 1978; Stevens et al, 1978). This is almost certainly due to the very brief viraemia of between 7 - 10 days that occurs 1 - 2 weeks prior to clinical symptoms.
The principal mode of transmission for the virus is the faecal - oral route, although subcutaneous inoculation may also lead to infection with little or no variation in resulting incubation time or concentration of viral shedding. The route by which the virus enters the liver remains unresolved, although it has been detected by specific mAbs and cDNA probes in various tissues including intestinal, lymph and kidney (Fagan et al, 1990), suggesting that replication in the jejunum prior to transmission via the portal vein may occur (Gust,

1988). HAV is shed in the faeces 3 - 10 days before the onset of clinical symptoms, which occurs after an incubation period of around 28 days. This shedding of virus occurs at approximately the same time as, or marginally before, an increase in ALT (alanine aminotransferase)levels, indicating liver damage. Communicability of the infection is at its highest during this period. The presence of HAVAg in the bile of infected chimpanzees during viral shedding indicates that this is the method by which virus passes from the liver to the gut (Schulman et al,1976).

1.1.3 Pathology

The infection manifests itself in varying degrees of severity. As has already been mentioned, in young children the condition may be totally symptomless, while in other cases symptoms such as nausea, jaundice, diarrhoea, dark urine, abdominal pain, malaise, fever and myalgia may occur to varying degrees. Unlike other causative agents of viral hepatitis, HAV is not known to cause persistent infections, although 10% of cases follow a protracted course of up to 9 months. In a very limited number of adult cases fulminant viral hepatitis A can occur (Rakela et al, 1978; Mathiesen et al,1980) potentially leading to coma and death.

It now seems certain that the symptoms of this infection are not as a result of a direct cytopathic effect of the virus, but mainly occur via a T - cell mediated immunopathological mechanism (Kurane et al, 1985). This can be shown by the lack of cytolytic effect of HAV on tissue culture fibroblasts (Valbracht et al, 1986), the increase in macrophages in the liver biopsies following infection and parallels with the recognized immunopathology of HBV infection.

1.1.4 Immune response and serological diagnoses

An increase in the total levels of serum immunoglobulins is common during acute HAV infections (fig. 1.1) and may include antibodies to HAV as well as to gut bacteria and other unrelated antigens. The reason for such a non - specific response is not known. The specific response to HAV however, is

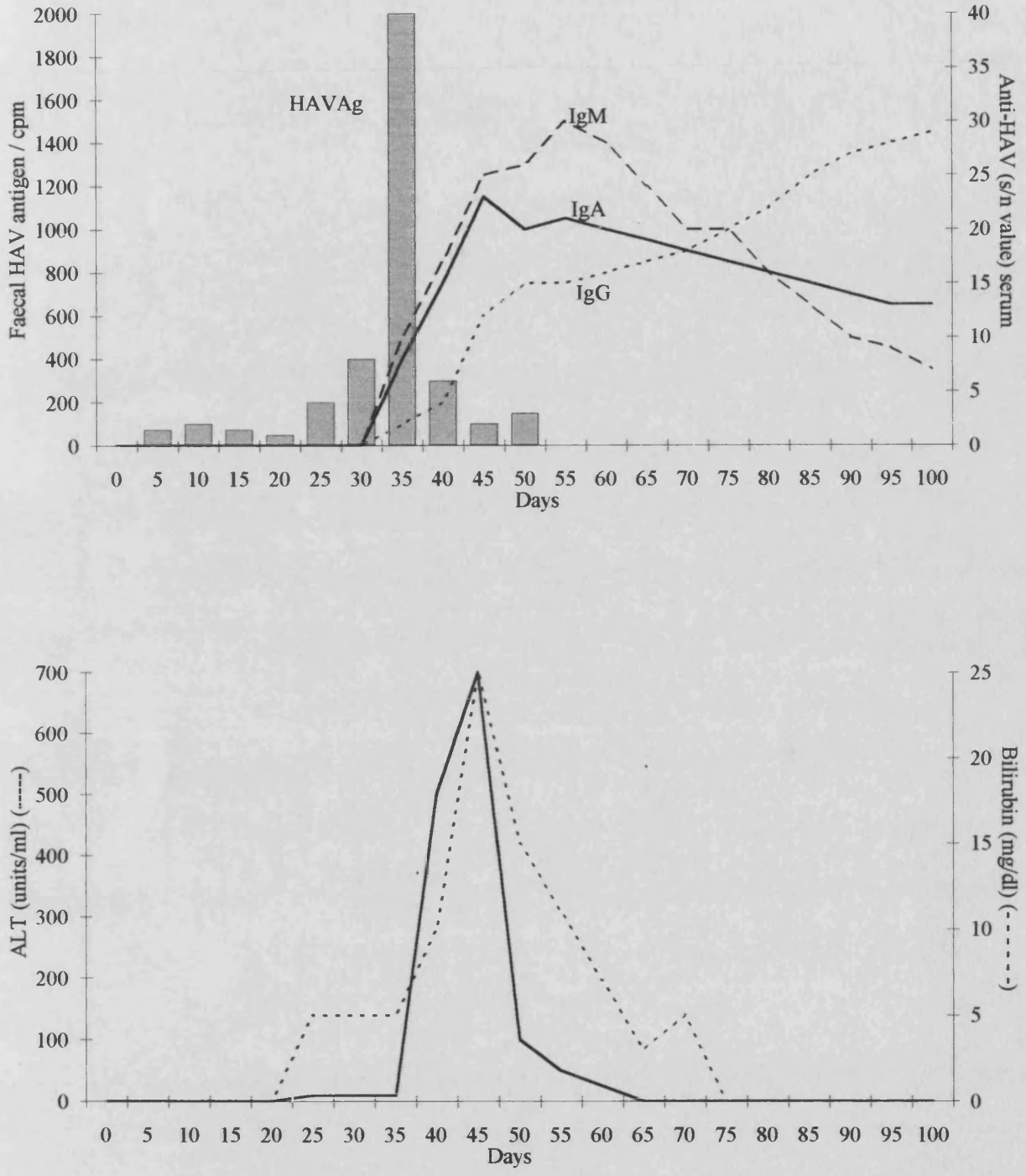


Fig. 1.1 Clinical course of HAV infection in a human volunteer given an oral inoculum of the MS-1 strain of virus.

certainly present by the onset of symptoms (Lemon et al, 1980; Rakela et al, 1977; Decker et al, 1979; Locarnini et al, 1977)) and reaches a peak titre 1 - 2 weeks later. A number of assays have been developed to detect these serum antibodies, including immunoelectron microscopy (Feinstone et al, 1973), complement fixation (Provost et al, 1975a), radioimmunoassay (Hollinger et al, 1975) and enzyme immunoassay (Locarnini et al, 1978). The IgM response is typically short - lived ,and was first detected by immunoelectron microscopy by Locarnini (1974) and subsequently by various serological assays (Locarnini et al,1977; 1979). IgM is replaced with IgG and IgA which first appear as part of the total immunoglobulin response. Serum IgG persists for long periods of time, perhaps for life, although levels may drop below those detectable by current methods (Ajdukiewicz et al, 1979; Villarejos et al, 1982). If challenged with HAV a second time, an immediate IgG response ensues, devoid of IgM, usually resulting in an asymptomatic infection (Villarejos, 1982). The success of passively administered immunoglobulin suggests that this IgG response to HAV is primarily involved in protection against a second challenge from the virus, and that it is more likely to be other immune mechanisms, such as interferon's, natural - killer cell activity and T - cell cytotoxity which is responsible for controlling a primary infection. Though IgA is known to be present in faecal samples from infected individuals, its role in immunity is unknown.

1.1.5 Therapy and prophylaxis

Treatment of HAV infections is usually supportive, with a recommendation of bed rest and a high calorie diet. It is probable that corticosteroids accelerate the recovery from late cholestasis (Gordon et al, 1984) although a small study using methyl - prednisolone indicated a substantially higher mortality in treated patients as opposed to those given a placebo (Gregory et al, 1976). The virus appears to be resistant to most established anti - viral drugs but various agents are being evaluated in tissue culture. Amantadine and ribavirin are two

showing sufficient promise to warrant clinical trials (Passagot et al, 1988). Interferons have also been shown to be effective in vitro (Vallbracht et al, 1984) which is of particular relevance since HAV is a poor interferon inducer (Vallbracht et al,1985).

Prevention of HAV infection is essentially dependent on high standards of hygiene particularly clean, chlorinated drinking water and the supervision of food and water handlers. Transmission within families, even in the western world, remains high with rates as high as 20%. Barrier nursing is not required in hospitalized cases and only in neonatal nurseries must particular care be taken. In general HAV is not considered to be an occupational hazard. In the laboratory, spillages represent a theoretical risk and therefore the use of cabinets to contain aerosols is justified. The 60 - fold concentration increase of HAV in shellfish from the surrounding water, as a result of the large throughput of oxygenated water, represents another area where hygiene methods can be improved. Presently the industry standard is a total replacement of water in shellfish beds every 2 -3 days. This is clearly insufficient, since studies have indicated that HAV particles can remain in these tanks for up to 5 days (Sobsey et al, 1988). Limited protection from infection by HAV, of around 3 months, can be conferred by the intramuscular injection of normal pooled immunoglobulin (NPIG). This relies on the prevalence of anti - HAV immunoglobulins in the normal pooled sera to protect the immunized individual. There is the possibility of spread of HAV by passive immunization, but the effects are highly attenuated and may indeed result in life - long immunity, though this must never be assumed. There have also been no recorded cases of infection from other organisms following use of this preparation since it is prepared by Cohn ethanol fractionation, which is one of the safest methods of preparing pooled immunoglobulins for passive immunization. This form of protection is normally only administered to individuals from non - endemic areas visiting regions with a high incidence of

the virus. In the UK. this is extended to any country outside Europe except U.S.A., Canada, Australia and New Zealand. In developed countries, post exposure passive vaccination is also recommended for household contacts during outbreaks.

The main concern with this form of protection is two - fold. Firstly this type of vaccination will only protect for 6 months, making it impractical for people who may experience multiple exposures. Secondly, as the prevalence of HAV - specific antibodies in the community is reduced, the efficacy of the immunization is compromised. Both of these problems have led to the need for an improved vaccine

The production of a vaccine, as with many other viruses (most notably poliovirus) has been approached in two ways. The first method is the preparation of a formaldehyde - killed virus, which at present is the only licenced form of HAV vaccine available.

There are however, problems associated with killed vaccines. Firstly the cost of production can be very high as a result of the poor growth of HAV in tissue culture making the immunization of the whole population impractical. Secondly, though an effective immunogen, booster injections are likely to be required periodically, which may not be necessary with an attenuated vaccine. Some doubt lies with the ability of killed vaccines (as with poliovirus Salk vaccine) to produce secreted IgA antibodies in the gut, which would insure complete neutralization of the virus and subsequently halt its spread. The formalin - inactivated Salk poliovirus vaccine has nevertheless proved to be highly effective in the countries of Western Europe where it has been exclusively used.

Clearly the most effective way of combating HAV in the long term is the introduction of a live attenuated vaccine, as has been so successful with other viruses. This would ensure life long effective immunity, would overcome many of the production costs involved with the current killed vaccine methods

(less antigen would be required per inoculaton and less often) and would, if necessary instil herd immunity through effective gut IgA production. There are however, problems associated with this type of vaccine, as has been shown with poliovirus Sabin 3 strain, where reversion to a neurovirulent form takes place in the gut and may then be excreted. Infection by neurovirulent poliovirus vaccine revertants only occurs, however in 1 in 1 million cases, and in the U.S.A. live attenuated vaccines are now the only form used.

There are two groups working on the attenuation of HAV, one at the Merck Institute working with strain CR326 and the other at the National Institutes of Health working with HM175. Both groups have concentrated on multiple passaging of the virus in cell culture.

Recent studies with strain CR326 have produced two pools of cell - culture variants named F and F' (Provost et al., 1986). F produced mild elevations in serum ALT in 3 of 12 marmosets tested, whereas F' produced no evidence of disease at all in 12 of 12 marmosets, yet produced detectable anti - HAV antibodies in 10 of the 12 animals. Both sets of marmosets were resistant to a challenge of 10^5 ID_{50} of wild type virus. In humans, F produced mild elevations of ALT in 9 of 25 tested, with F' producing detectable levels of anti - HAV in 10 out of 11 subjects, with no sign of disease in any. Passage 32 of HM175 has shown to be attenuated for marmosets, producing only slight increases in ALT levels in one of four animals immunized.

Though these results look very promising, certain questions have to be answered; can the oral vaccine produce a protective response, is the vaccinated subject resistant to oral and intravenous challenge, is the virus subsequently secreted in the faeces, can the vaccine revert to virulence and will liver function tests be unavoidable?

There are other methods of vacccine production available. Molecular cloning of the HAV genome, and subsequent surface probability plots and homology comparisons have allowed the production of small oligopeptides containing

candidate neutralization sites (Emini et al., 1985). A synthetic peptide containing amino acids 11-25 of VP1 has been used with laboratory animals and has produced neutralizing antibody. The response however, was highly variable between animals and will require further work to improve immunogenicity. HAV encoded proteins have been expressed as fusion proteins in E.coli (Ostermayr et al., 1987; Ross et al., 1988; Johnstone et al., 1988) and in insect cells (Harmon et al., 1988) Two of these proteins containing VP1 sequence produced a response to VP1 but failed to produce a response to whole virus (Ross et al., 1988; Johnstone et al., 1988). A Trp/VP1 fusion protein however, was able to prime the immune response such that an immune response was elicited against an otherwise subimmunogenic concentration of whole virus(Johnstone et al., 1988). As it is now widely accepted that the neutralizing sites on the HAV capsid are conformationally formed, it is likely that expression of an empty capsid may be necessary to produce an effective immune response. Indeed, the maturation of virus structure associated with encapsulation of viral RNA may still be required to elicit an acceptable response.

1.1.6 Virus structure

The Hepatitis A virus is a small, non - enveloped virus of the family Picornaviridae. It was previously classified in the genus enterovirus, but certain unique characteristics such, as stability in high temperatures and its tendency to establish persistent infections *in vitro* (Provost and Hilleman, 1979) casts doubt on this classification. With the publication of sequence data (Linemayer et al, 1985; Najarian et al, 1985; Baroudy et al, 1985) the comparison between HAV and other picornaviruses was extended to genomic organization (Palmenberg, 1987, 1989) and it was realized that while homology within genera is high, homology between genera is considerably less, with HAV sharing less homology than is present between the other genera. On this basis HAV has now been placed in its own genus, hepatovirus

(Francki,1991). Mature virions appear spherical (see fig.1.2), are approximately 27nm in diameter and have icosahedral symmetry (Feinstone et al, 1973; Siegl et al, 1982). The sedimentation coefficient of the virus is 156S and the density is 1.32 - 1.34g/cm^2 in caesium chloride (Siegl et al, 1981; Lemon et al, 1985), with minor populations of viruses having densities of 1.27 - 1.32g/cm^2 and 1.39 - 1.50g/cm^2 (Coulepis et al, 1982). The 'light' viruses probably represent empty capsids (Tratschin et al, 1981; Gauss - Müller et al, 1986), mature virions associated with lipids (Lemon et al, 1985) or defective interfering particles (Garelick et al, 1988). 'Heavy' particles are thought to have taken up CsCl during centrifugation (Cooper et al, 1978). The proportions of these minor populations may reflect the strain under investigation and the purification methods employed. The stability of HAV is similar to that of the other picornaviruses except in its ability to withstand relatively high temperatures (see Table 1.3). The virus is also stable at a pH of 3.0 (Siegl et al, 1984) and is resistant to many lipid solvents (Provost et al, 1975). HAV is however, readily inactivated by formaldehyde, a fact which has been utilized in the production of killed active vaccines (Provost and Hilleman, 1978). When purified virus was subjected to SDS - PAGE, three structural proteins were revealed (Coulepis et al, 1978) with apparent molecular weights of 34, 25.5 and 23 kDa, consistent with the three main structural proteins of better characterized picornaviruses. Later reports identified a putative fourth protein with an approximate weight of 7 - 14kDa (Coulepis et al,1980; Tratschin et al, 1981). Using nucleotide sequence data, and alignments with known picornavirus structures, a more detailed analysis of the capsid proteins has been possible (Linemayer et al, 1985). The majority of the proposed gene locations for the capsid proteins and cleavage junctions have now been confirmed by amino acid sequencing (Linemayer et al, 1985; Gauss - Müller, 1986) and studies with synthetic peptides (Wheeler et al, 1986) with only the VP1/2A junction having been modified as a result of protein

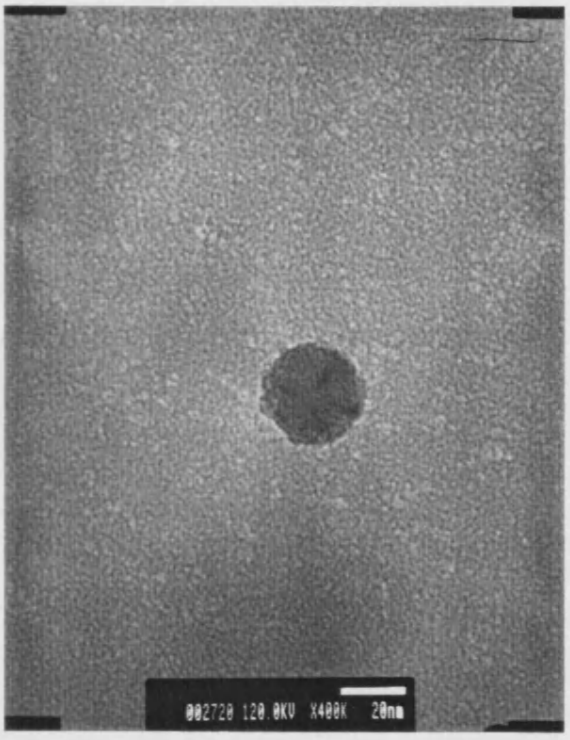


Fig 1.2 Electronmicrograph of a Hepatitis A virus particle fixed and stained in phosphotungstic acid (x 400,000)

	HAV	ENTERO (PV1)	RHINO (HRV14)	CARDIO (EMCV)	APTHO (FMDV A12)
SEROTYPES	1	>70	>130	2	7
STRAINS	13			6	53
1° HOST	Humans, other primates	Humans, other mammals	Humans, other mammals	Mice, other mammals	Cloven-footed, other mammals
TISSUE TROPISM	Narrow	Narrow to wide	Narrow	Wide	Wide
PRIMARY HABITAT	Liver	Gut	Upper respiratory tract	CNS, heart	Generalized
SENSITIVITY					
acid (pH3)	Stable	Stable	Labile	Stable	Labile
heat (60°C,60')	Stable	Labile	Labile	Labile	Labile
guanidine	Resistance	Sensitive	Sensitive	Resistance	Resistance
disoxaril	Resistance	Sensitive	Sensitive	Resistance	Resistance
BIOPHYSICAL					
Buoyancy density (g/cm^3 CeCl)	1.32-1.34	1.34	1.39-1.42	1.34	1.43-1.45
Sedimentation coefficient	156-160	156-160	149	156	142-146
VIRION PROTEINS (M_R) [kDa]					
VP1	33.2	33.5	32.4	31.7	23.3
VP2	24.8	30	28.5	29	24.7
VP3	27.8	26.4	26.2	25.1	24.3
VP4	<2.5	7.4	7.2	7.2	8.5
VPg	2.4	2.3	2.4	2.2	2.6-2.7
GENOME					
length (kb)	7.48	7.44	7.21	7.84	8.4
%(G+C)	38	47	40	50	43
Poly(C)'	-	-	-	+	+
Poly(C+T)'	+	-	-	-	-
Similarity	HAVs	Entero,Rhino	Rhino,Entero	Cardio	Apthos

Table 1.3 Biophysical characteristics of Hepatitis A Virus compared with other picornaviruses.

sequencing. The predicted molecular weights for the four capsid proteins are 33.2kDa (VP1), 24.8kDa (VP2), 27.8kDa (VP3) and 2.5kDa (VP4). Contradictory SDS-PAGE sizing of the three largest proteins, particularly VP2 and VP3 was explained by Wheeler et al (1986), who noted that the migration of VP2 was significantly affected by the presence of urea in the gel system. This was confirmed by direct comparison of the two systems using immunoblotting techniques using antisera to VP2 and VP3 (Ross, 1988). As with the other picornaviruses some virus particles contain a VP0 protein, which is the precursor protein for VP2 and VP4 (Gauss - Müller et al, 1986; Ross, 1988). Though this would suggest that VP4 is generated by the cleavage of VP0, though further investigation is required to confirm this. One unanswered question is the size of VP4. Consensus sequences have indicated that there is a myristylation site similar to that of other picornaviruses at gly- 7. This would leave a peptide of only 17 amino acids with a molecular weight of 1.7 kDa which has yet to be detected in virions or infected cells.(Chow et al, 1987; Palmenberg, 1989)

1.1.7 Antigenic structure

Currently the understanding of the antigenic structure of HAV is very basic. This is because attempts to characterize the relevant regions of the capsid have been impeded by the slow and generally non - cytopathic replication of HAV. This results in a lack of X - Ray crystallographic data which is available to other picornavirologists. Some progress has been made with the use of neutralization escape mutants and peptide immunogens, coupled with computer alignments with other picornaviruses.

Although only very few HAV strains have been investigated in depth, it is evident that there is no significant antigenic variation between them. This can be exhibited in the classical cross neutralization experiment, for instance, between strains HM175 recovered from a human in Australia and PA21 recovered from a New World Owl monkey, where sera raised to one strain

successfully neutralized the other (Lemon and Binn, 1983). In contrast there are marked differences in the RNA sequence in the P1 region of these viruses which suggests that these viruses are genetically distinct from one another (Lemon et al., 1987). This all suggests that the constraints on HAV to maintain this antigenic structure are extremely strong.

There is now considerable evidence that the antigenic sites on the HAV capsid are created during the assembly and folding of the capsid proteins, rather than being sequential immunogens (Gerlich and Frosner, 1983). This can be partly demonstrated when attempting to detect denatured capsid proteins with neutralizing antibodies on a western blot, where staining does not occur. (Hughes, 1985) Moreover it has been shown that individual capsid proteins, even those bearing parts of the immunogenic region, elicit very little antibody reactive to native capsid. Although the amino acid sequence of HAV and poliovirus type 1 have little in common, surface probability profiles of the N-terminal halves of the two peptides indicate that there may be some higher order similarites (Emini et al., 1985; Najarian et al., 1985). Specifically VP1 residues 89 - 103 that form the immunogenic "C3 loop" of polio (Hogle et al., 1985; Minor et al., 1986; Wychowski et al., 1983) may be conserved in HAV. Furthermore the HAV VP1 residues 11 - 25, selected because they correspond in the surface probability profile to an immunogenic region of poliovirus VP1 sequence, elicit a low titre of neutralizing antibody in rabbits (Emini et al., 1985). These residues in poliovirus, however are not on the capsid surface but may only be externalized when the virus binds to its cellular receptor. It is likely, therefore that in HAV these residues do not represent a major immunogenic site. Various other attempts have been made to induce an antibody response from peptides expressed in E.coli. The N-terminal 60 amino acids of HAV VP1 when expressed as a fusion protein with E.coli β - galactosidase does appear to be weakly antigenic (Ostermayr et al., 1987). Likewise, expression of the entire VP1 polypeptide with the carboxy-terminal

46 amino acid of VP3 as a TrpE fusion protein is capable of producing an antibody response to denatured VP1 but not intact virus (Johnston et al., 1988). It is interesting to note that this second polypeptide is capable of priming for a neutralizing antibody response to subimmunogenic quantities of intact virus, and therefore probably contains relevant T - cell epitopes.

As has been mentioned, one of the more important ways of determining the neutralization sites on the HAV capsid is the induction of escape mutants. These are viable virus mutants which arise as a result of replicating HAV in the presence of neutralizing antibodies. As a result, they have altered neutralization sites such that the antibodies are no longer able to bind. By sequencing their genomes the position of these sites can be established and therefore the position of wild type virus neutralization sites.

Two sites of interest have so far been identified, a G to C change at position 1677 which results in an amino acid change from Asp70 to His on VP3 and a second, apparently a less effective escape mutation, occurs at position 2512 with a C to T change resulting in a Ser to Leu alteration at amino acid 102 of VP1 (Stapleton and Lemon, 1987). These results suggest that Asp70 on Vp3 represents an important immunogenic site on the HAV capsid and changes at this point produce a conformation that the monoclonal antibodies are unable to bind to. The alternative, but less likely possibility is that the amino acid change stops the monoclonal antibodies binding elsewhere on the capsid by global conformational changes. This is made more unlikely by the known clustering of escape mutations around the cellular binding site on the poliovirus and rhinovirus. Unfortunately for simplicity, the crystal structures of other picornaviruses suggest that the two sites mentioned above are in fact distant from each other, VP1-102 lying near the five-fold axis of symmetry and VP3-70 lying at the three-fold axis. There are therefore two possible explanations at present, firstly that the structure is not sufficiently similar to other picornaviruses to compare, or that indeed the binding of monoclonal

antibodies at sites distant from the neutralization site can exert changes. This second explanation has been shown to be true for FMDV virus.

The neutralization mechanism for HAV is at present unknown.

1.1.8 Molecular Virology

Early observations that HAVAg exists exclusively in the cytoplasm of an infected cell provided evidence that HAV was an RNA virus and did not reverse transcribe its genome into DNA for integration into the host cells genome (Provost et al, 1975; Mathieson et al, 1977). Staining of the particles with acridine orange also suggested that the viral genome was single stranded. (Provost et al, 1975). In 1981 Coulepis et al showed that the genome was of single - stranded RNA containing poly(A). The nucleic acid sedimented at 35S, with a bouyant density of 1.64g/cm^2.

More recently, the cloning of the HAV genome as cDNA from virus purified from the liver of an infected marmoset, has allowed more detailed analysis of its makeup (Ticehurst et al,1983). The complete sequence was subsequently determined (Baroudy et al, 1985; Cohen et al, 1987) and this remains the only wild type sequence published to date. Several sequences, however, have been established from virus purified from tissue culture after differing numbers of passages (Najarian et al, 1985; Linemayer et al, 1985; Venuti et al, 1985; Ross et al, 1986; Cohen et al, 1987; Ostermayr et al, 1987; Jansen et al, 1988).

Cloning and restriction mapping of the genome have indicated that it is approximately 7500 nucleotides long (Ticehurst et al, 1983), consisting of a 5' non - coding region of 734 nucleotides, an open reading frame encoding 2227 amino acids followed by two termination codons, a second, 60 base non - coding region and a 3' poly (A) tract (Baroudy et al, 1985; Linemayer et al, 1985; Najarian et al, 1985). By comparing the predicted amino acid sequence for HAV to that of poliovirus type 1 in homology computer programs (Najarian et al, 1985), a complete putative polyprotein cleavage map has been

assembled. Subsequently other programs have been used to predict structural and hydrophilic properties of the proteins encoded by the genome and therefore to predict possible cleavage sites, most of which are atypical for picornaviruses.

In fig 1.4 the genomic organization of HAV is compared with that of poliovirus using the L434 nomenclature of Rueckert and Wimmer, (1984). The nomenclature divides the genome into regions P1, P2, and P3. The P1 proteins (1A - 1D) represent structural proteins while P2 (2A - 2C) and P3 (3A - 3D) are involved in virus replication.

The localization of the genes encoding virus proteins has been partly achieved by amino acid sequencing of purified structural proteins. Linemayer et al, 1985 showed that the N - terminus of VP1 mapped to nucleotide 2208 which is in agreement with amino acid sequence results from reverse - phase high pressure liquid chromatography performed by Gauss - Müller et al, 1986. Gauss - Müller et al (1986) also used this method to sequence the N - terminus of VP2 to give the same result as that obtained by Cohen et al, (1987).

The localization of the HAV structural proteins have been further characterized by successfully reacting sera raised to synthetic oligopeptides representing specific areas of the P1 region with immunoblotted HAV proteins.(Wheeler et al, 1986 b) The location of VPg was established by immunoprecipitating the protein from purified virus with antisera raised against oligopeptides representing the predicted sequence of VPg.

The non-coding regions of the HAV genome have some notable features. There is a high proportion of pyrimidines (C+T) near the 5' terminus of the genome (95% of nucleotides 99-138 and 68% of nucleotides 31-249) and it is also here where there is most conservation between strains (>95%) This region is considered to be analogous to the poly(c) tract seen in cardioviruses.

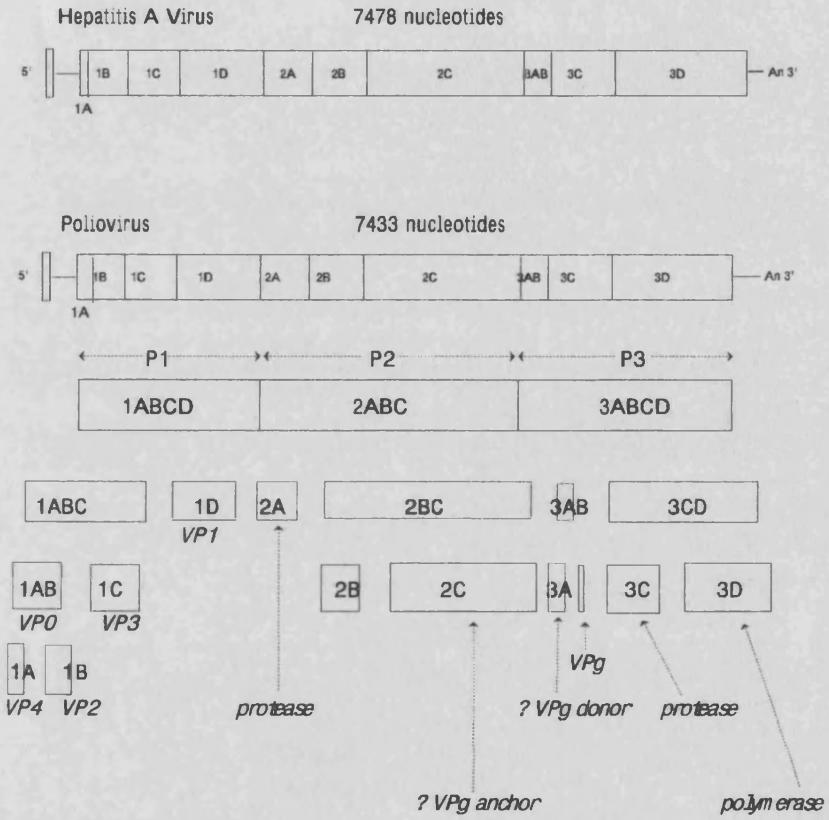


Fig 1.4 Genomic organization for hepatitis A virus and poliovirus (L343 nomenclature) and the protein processing map of the poliovirus polyprotein. Virion RNA is covalently linked to a protein, VPg at the 5' end and has a poly (A) tract. Protease 2A cleaves between P1 and P2. Protease 3C cleaves all other sites exept for 1A/1B, which is autocatalytic. P1 are structural proteins whilst P2 and P3 perform replicative functions.

In contrast, the 3' terminus of the HAV genome has the highest percentage differences between strains, at about 20%, and it is this portion of the sequence which is most often used to differentiate strains.

As with other picornaviruses, the sequence and secondary structure of the 5' non - coding region of HAV appears to play an important role in translation. It has been known for some time that the 5' NTRs of picornaviruses are well conserved, despite the high error frequency normally associated with RNA viruses. This suggests that these areas are important for replication, with minor base changes in the region having major effects on replication and virulence, as has been demonstrated with poliovirus (Racianello and Meriam, 1986; Kuhn et al, 1988; Nomoto and Wimmer, 1987). It is likely therefore, that picornaviruses have a number of control elements in this region which effect replication, translation and encapsidation. Picornavirus RNA is uncapped and contains many noninitiating AUG codons. By a series of experiments on EMCV, which involved testing the inhibitory effects of cDNA to various portions of the 5' NTR, it was found that interfering with the extreme 5' end of the genome did not effect translation, whereas hybrdization of the cDNA strands to areas downstream in the putative ribosome entry site did (Shih et al, 1987). This internal ribosome entry site (IRES) was further demonstrated by investigating the expression of a polycistronic mRNA containing non - overlapping open reading frames (ORF)(Jang et al, 1988; Jang et al, 1989; Pelletier and Sonenberg, 1988). The 5'NTR of either EMCV or poliovirus was inserted between ORF, resulting in the cap independent translation of the adjacent downstream ORF. In frame mutation of this picornavirus NTR resulted in extremely poor translation of the adjacent sequences.

Work is continuing to identify the IRES in various picornaviruses and to investigate its structure. The sequencing of the HAV 5' NTR has indicated that the same structures exist as with other picornaviruses. Brown et al, (1991)

have used a phylogenetic approach in developing a secondary structure for the 5' NTR. According to this model the 5'NTR contains six major domains, including a hairpin loop at the 5' terminus, a long single polypyrimidine tract, and two large stem loop structures with multiple covariant mutations located between bases 324 and 692. The stem loops share several motifs with EMCV. A rabbit reticulocyte lysate system was used to investigate expression under various reduced versions of the 5' NTR of HAV. It was established that removal of nucleotide (nt) 1 to 354 had little effect on translation, while deletion up to nt 457 resulted in decreased translation. Deletion up to nt 533 completely abolished translation . Further deletion, to nt 633 increased translation again to a level greater than the full - length RNA. . This suggests that the sequence 3' to base nt 355 plays a vital role in the translation of genomic length RNA.

1.1.9 Replication of HAV in Tissue Culture

It was not until 1979 that Provost and Hilleman first isolated HAV. This delay was due to problems associated with culturing the virus, such as its unusually long replication time, the resulting contamination from other viruses, and the difficulty of detecting the virus in the absence of cytopathic effect.

The problem of slow replication still remains, but the detection of HAV has improved considerably, with the immunofluorescence assay of Mathiesen et al, (1977) which Provost and Hilleman used. HAV strain CR326 which had been passaged multiple times in *Sanguinis mystax* and *Sanguinis labiatus* marmosets was inoculated into explant cultures of *S.labiatus* liver tissue. Within 8 days cells showed evidence of infection, and after 23 days the majority of cells showed immunofluorescent staining of HAVAg. Following this the virus was passaged in FRhK-6 cells but with no cytopathic effect. Subsequently, others demonstrated adaptation to cell culture without prior passaging through marmosets. (Frosner et al, 1979b; Balayan et al, 1979; Daemer et al, 1981; Locarnini et al, 1981). Flehmig et al (1980; 1981)

established a line of persistently infected FRhK-4 cells which eventually resulted in the realization that, unlike other picornaviruses, persistent infection with HAV can result in constant low level production of HAVAg with no effect on host cell morphology and synthesis of macromolecules (Gust and Feinstone, 1988). Only recently have strains been produced, through multiple serial passaging, that have sufficiently short replication times to allow full studies of the kinetics of HAV replication.

In 1985 another important breakthrough in the study of HAV took place. Venuti et al reported the isolation of an HAV strain which caused cytopathic effect after 7 - 10 days, a result that was repeated by Wu and Co - workers (1986) in A549 cells infected with faecal samples from naturally infected humans.

As confirmation that c.p.e. in HAV - infected cells is due to HAV and not contaminating viruses Anderson et al, 1987 reported c.p.e. in BS-C-1 cells, which could be neutralized with HAV - specific monoclonal antibodies and convalescent sera from experimentally infected chimpanzees. In addition, the infective agent was shown to be extremely stable in conditions where only HAV would be expected to survive. The reason for the cytolytic nature of these strains remains unclear, but cellular factors and culture conditions (such as serum concentration and the age of the cells) are likely to be important .

Several methods have been used to assay the infectivity of a virus sample. The first method of assay, immunofluorescence of infected monolayers, has since proved to be too time consuming and was eventually replaced by simpler radioimmunoassays, techniques which have been refined for greater and greater sensitivity (Deinhardt et al, 1981; Siegl et al, 1984; Vallbracht et al, 1984; de Chastonay and Siegl, 1987). Lemon et al (1983) described a modification of the plaque assay which has become the standard infectivity assay for HAV. Cell monolayers are infected with serial dilutions of the test sample and covered with a media/agarose overlay as in a standard plaque assay.

In order to visualize the plaques however, the monolayer is fixed and stained with HAV - specific polyclonal antibody followed by [^{125}I] - protein A. Though much simpler plaque assays are now also available for infectious HAV, the radioimmunofocus assay has the principle advantage of taking only 6 days to visualize plaques, whereas the plaque assay can take as long 14 days.

With the isolation of HAV in culture well established, several groups began to look at the kinetics of HAV replication. Initially this was simply to look at the rate of antigen production, but eventually moved on to characterizing other aspects of replication by recording the synthesis of virus - specific molecules (Locarnini, 1981; 1982). In these studies, specific molecules were detected using the double - label co-electrophoresis and subtraction technique (Westaway, 1973), in which virus infected cells are labelled *in vitro* with [^{3}H]-leucine whilst uninfected cells are labelled *in vitro* with [^{14}C]-leucine. The two are then subjected to SDS-PAGE analysis, a labelled protein profile obtained by counting the gel slices and finally the uninfected profile subtracted from the infected profile to indicate virus specified proteins. Within 6 hours proteins and nucleic acids were detected with a similar size distribution to that of poliovirus - infected cells. This same macromolecular profile was also demonstrated from cells infected with HAV RNA, providing the first evidence that viral RNA is in fact infectious.

Unlike poliovirus however, recent studies using incorporation of radiolabelled amino acids into viral proteins (only recently possible with the advent of fast growing strains) suggest that there is a significant lag between virus protein production and their incorporation into virions. Improvements in infectivity assays have also allowed more detailed analysis of replication, with Wheeler et al.(1986a) making the important observation that HAV is not efficiently uncoated in tissue culture, with a substantial proportion of the inoculum being recovered for up to 12 hours, again in contrast to the behaviour of poliovirus.

Though difficulties in detecting viral RNA have hindered studies of replication, cDNA probes and the radioimmunofocus assay have established that it is the synthesis of RNA which is the rate limiting step in the replicative cycle of the cytopathic strain HM175 in BS-C-1 cells; with no apparent lag phase between synthesis of RNA and production of protein (Anderson et al, 1987). Anderson et al (1987) also confirmed the observation that HAV uncoats very slowly and that this is not due to slow penetration through the cell membrane. Preliminary studies have shown that the virus undergoes a series of changes before uncoating, with the virion becoming permeable to neutral red within 2 hr, although 160S virions can be recovered up to 8 hr after infection with no detectable RNA synthesis (Anderson et al, 1988). Studies also suggest that the restricted RNA production is as a result of the high efficiency of RNA packaging within virions (Anderson et al, 1988). Another notable difference between poliovirus and HAV is that during poliovirus replication, progeny RNA is controlled such that there are large pools of intermediates and polyribosomal RNA. In contrast, during HAV replication progeny RNA is continuously being packaged into virions. As a result the pool of RNA available for replication is depleted (Anderson et al, 1988). Fig.1.5 shows a model for the replicative cycle of HAV. Persistent infection in tissue culture is thought to be as a result of a reduction in RNA synthesis (de Chastonay and Siegl, 1987), although a sub - genomic RNA species which may be associated with persistence has also been detected.

There is now a suggestion that defective interfering particles (DI) may play a part in persistence in cell culture (de Chastonay and Siegl, 1987; Garelick et al, 1988). These are HAV particles which have large deletions in the genome. They are not infectious but can inhibit infection by viable virions. DI particles have been identified in cell culture infected with various viruses and have been associated with persistence in Foot - and - Mouth disease infections in culture. They have also been detected in cell cultures infected with HAV at high

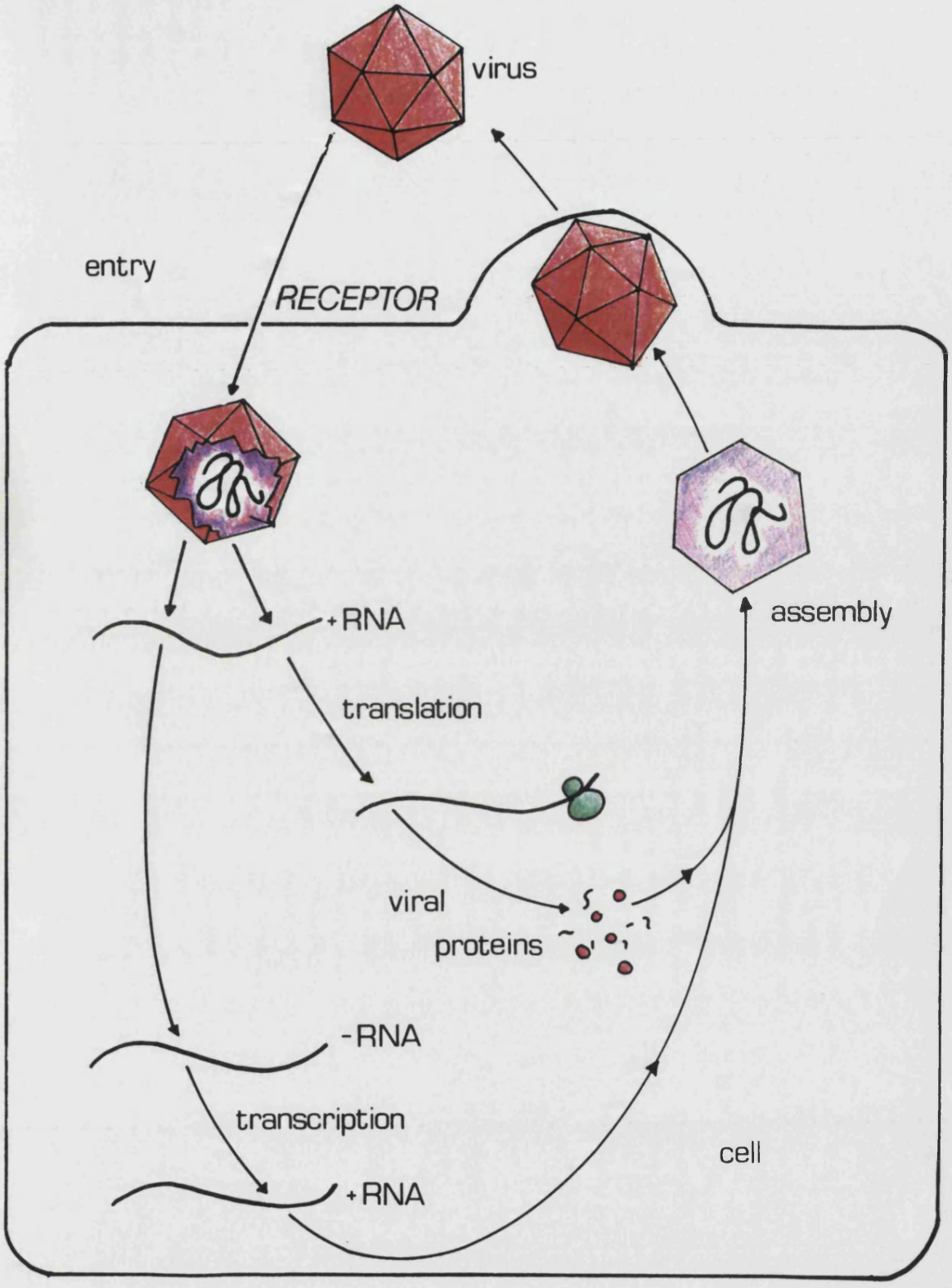


Fig 1.5 Replicative cycle of Hepatitis A Virus.

multiplicities of infection (m.o.i.), but as persistence in HAV has not been associated with high m.o.i. the significance of DI particles in HAV culture is unclear.

1.2 VIRUS CELLULAR RECEPTORS

1.2.1 Introduction

The plasma membrane of cells represents, amongst other things, a barrier to invading organisms such as parasites and viruses. Even though it is impermeable to ions and small molecules as well as proteins and polypeptides, it is clear that viruses do transport their genomes and accessory proteins into the cytosol of the target cell.

A multitude of different methods have evolved for the transport of vital compounds and ions in and out of the cell and to facilitate communication with other cells. Since the plasma membrane has to be dynamic to do this, constantly cycling the molecules on its surface, it was considered likely that viruses would utilize these molecules to gain access to the cell. Though this point is now irrefutable, the details of which molecules different viruses use, and how they use them are, in many cases, not understood. One thing is clear, the future of virus cellular receptor and cell biological research are likely to be closely linked. Research in this area has always been difficult. This is, in part, because of the problems associated with the techniques involved. Identification of the receptor is often hampered by weak affinities between virus and receptor, low receptor copy numbers on the cell surface, viruses utilizing more than one receptor type and the high degree of non - specific associations. The sensitivity of assays has also presented a problem, since a phase of replication prior to amplification of viral - associated molecules is often being measured.

Nevertheless with improvements in methods such as enzyme immunoassays and radiolabelling of virus, interest in the field continues. Indeed in some cases clinical applications of receptor identification and function are being utilized.

1.2.2 Cellular Endocytosis of Viruses

Many viruses including alphaviruses, orthomyxoviruses, paramyxoviruses, rhabdoviruses, retroviruses, herpesviruses and a number of nonenveloped viruses use the endocytotic pathway to gain entry to target cells (Dales et al., 1973; Helenius et al., 1980; Matlin et al., 1982a,b). Morphological studies indicate that this usually occurs by receptor mediated endocytosis in clathrin-coated pits. Work with Semliki Forest Virus (SFV) suggests that the uptake of virus by this path is very similar to that used by physiological ligands. At 0°C virus is bound evenly over the surface of the cell. Upon warming to 37°C viruses are relocated to coated pits and internalized in clathrin-coated vesicles. This sequence of events is not induced by the virus, is independent of multiplicity and can be blocked by anti-clathrin antibodies. Though the use of coated vesicles is common amongst viruses, some such as Sendai and Influenza, occasionally utilize non - coated vesicles resembling phagosomes as a portal of entry (Dourmashkin and Tyrell, 1974; Matlin et al., 1982a).

Entry of viruses into endosomes results in a vital sequence of events to enable successful infection of the cell. During normal function of endosomes, the acidity of the contents is gradually raised from about pH 6.2 in early endosomes, to around pH 5.0 in the late endosomes. This ensures the disassociation of physiological ligands from their receptors and, where appropriate, the down regulation of receptors by degradation. This reduction in pH results in a change in capsid conformation, different viruses undergoing this change at different pH's . If, by the use of acidotropic weak bases such as amantadine, the pH is not allowed to fall, internalization does not occur. The use of endosomes also ensures that the virus is finally released into the cytosol in the perinuclear space where replication may begin.

1.2.3 Entry of Enveloped and Non-Enveloped Viruses into the Cytosol

Many enveloped viruses fuse with the membrane during penetration of the target cell. This interaction takes place initially by way of a spike glycoprotein. In general these are oligomeric transmembrane molecules with a total molecular weight of between 200 - 400kDa, with the N - terminus on the external side of the membrane. It is this extracellular portion of the spike which represents the bulk of the glycoprotein, so-much-so that it is often visible under electron microscopy. Each virus particle contains 80 or more spikes, but it is unclear whether more than one at a time is required for infection. Many of these 'fusion factors' have covalently bound palmitic acid and N - linked sugars. The N-linked sugars are implicated in the correct folding of the glycoprotein and its transport to the surface, whilst the role of palmitylation is not clear. The fusion glycoproteins are subjected to proteolytic cleavage late in the secretory pathway, mediated by cellular proteases, which reveals hydrophobic N-termini, important for fusion in at least orthomyxoviruses, paramyxoviruses, coronaviruses and retroviruses (Choppin and Compans, 1975; Sturman et al., 1985; White et al., 1983). This proteolytic cleavage also occurs in SFV p62, but has been shown to be unnecessary for cell infection, whereas with Rhabdovirus G proteins there is no cleavage at all. Though there are obvious functional similarities between many fusion proteins, there is rarely any sequence homology, suggesting that the fusogens do not share a common ancestor.

One of the best characterized, and also one of the most effective fusogens is the haemagglutinin (HA) from the influenza virus, and as such represents an excellent model to describe entry of enveloped viruses into cells by membrane fusion.

The structure of the extracellular portion of HA has been determined by X-ray crystallography in its inactive neutral pH form (Wilson et al., 1981) and its synthesis has been described. It has been found to be a trimer (3 x 84kDa) in

which each monomer consists of two polypeptides, HA1 and HA2. HA2 is primarily involved with fusion of the virus and cell membranes, whereas HA1 is involved in binding the virus to its cellular receptor, sialic acid. It is not essential for sialic acid residues to be present on the target cell surface for fusion, but it improves integration of the two membranes . The fusion of influenza membrane and its target cell membrane is pH dependent (Stegmann et al., 1988), requiring a drop in pH, and subsequent conformational changes in the HA trimer. As the pH drops, the most exposed domains of the HA1 molecules disassociate, revealing a hydrophobic face which is made up, in part, of the N-termini of the HA2 molecules. Due to the hydrophobicity of the exposed face, the HA trimer is partially inserted into the target membrane and this in turn brings the two membranes into extremely close proximity. There is a resulting local dehydration between the opposing surfaces, and the two bilayers are able to flip and fuse, releasing the virus nucleocapsid into the cytosol.

There is still some doubt over the validity of this model however, particularly since there are known to be variations with other virus families. Some viruses require the presence of cholesterol in the membrane for effective infection (Kielian and Helenius, 1984), whereas others are pH independent and do not require an acid environment to fuse.

Fusion of virus and cellular membranes can occur at the cell surface without the use of endosomes if the medium is acidified, but the virus may become trapped between the plasma membrane and the cytoskeleton and fail to infect the cell.

The method by which non-enveloped viruses gain entry to the cytosol, without fusion with the membrane, is far from clear but probably involves a conformational change in the virus capsid. In polioviruses the low pH environment of the endosome leads to the loss of the VP4 protein and viral RNA. It seems likely that with the extrusion of the myristylated (and consequently highly

hydrophobic) VP4, the virus is aided in its translocation across the endocytic membrane. (Paul et al., 1987; Chow et al., 1987) The conformational change, which may be inhibited by weak bases, also reveals the amino terminus of VP1 which helps to attach the virus to lipid vesicles (Hogle et al., 1988) and may also disrupt the pentamer-pentamer interaction of the virus capsid, opening a portal by which the virus RNA and VP4 may exit (Rossman et al., 1985). The myristylation status of VP4 in the Hepatitis A virus however, is still unresolved and this may have a bearing on the infectivity of the virus.

1.2.4 Viral Cellular Receptors

Whether a virus is enveloped or not, to infect a cell it is necessary for it to bind to a component of the cell surface to ensure that the virus is sufficiently close for internalization events to take place. The use of specific molecules on the surface of target cells also ensures that the virus associates with, and infects the correct cell type. In short the cellular receptor or viral attachment protein (VAP) for any particular virus also confers an initial cellular tropism upon it.

Viruses can bind to an array of cell membrane components, including proteins, glycoproteins, lipids, glycolipids and oligosaccharides. With the large range of possible cellular receptors available, (see Table 1.6) it is not surprising that a bank of different initial virus -cell interactions has appeared, which are largely dependent upon the receptor that is utilized.

Some virus groups have a high specificity for certain receptor molecules which are only present on a limited number of cell types, such as HIV - 1 and 2, which utilize the CD4 molecule on the monocyte-macrophage cell lines, and the Epstein - Barr virus, which utilizes complement receptor 2. Though cellular tropism is not only determined at the receptor level, it is true to say that in many cases the possession of particular cell surface molecules can often determine cell type specificity.

DNA VIRUS	HOST CELL RECEPTOR	BINDING DOMAIN OF RECEPTOR	BINDING DOMAIN OF VAP
Papovaviridae *Polyomavirus* Polyomavirus	Sialyloligosaccharides (Fried et al, 1981)	NeuAcα2,3 Gal β1,3 GalNAc (Fried et al, 1981)	
Adenovirus *Mastadenovirus* Human Adenovirus	Class I HLA MHC molecule (Chatterjee and Maizel, 1984)		Residues 1-141 of E3gp (Chatterjeee and Maizel, 1984)
Herpesviridae *Cytomegalovirus* Human cytomegalovirus			Residues 204-297 of H301 α - domain gene product (Bech and Barrel, 1988)
Lymphocrytovirus Epstein-Barr virus	C3d receptor CR2 (CD21 of B lymphocytes) (Fingeroth et al., 1984)		Residues 21-29, 372-378,of gp350 (Nemerow et al., 1987). Residues 21-30,219-222, and 372-460 of gp350 (Tanner et al., 1987) Residues 21-29 of gp350 (Nemerow et al., 1989)
Poxviridae *Orthopoxvirus* Vaccinia virus	Epidermal growth factor receptor (Eppstein et al, 1985)		Residues 71-80 of VGF protein (Eppstein et al, 1985)
Hepadnaviridae Hepatitis B virus	Hepatocyte receptor for polymerized serum albumin via albumin (Machida et al, 1984) Hepatocyte receptor for polymeric IgA (Neurath et al, 1986) Sialoglycoptroteins (Komai et al, 1988)		PreS portion of Env protein (Machida et al, 1984) Residues 21-47 of PreS1 portion of Env protein (Neurath et al, 1986) PreS1 sequence of large protein (hepatocyte receptor) and preS2 sequence of middle and large S proteins (polymerized serum albumin) (Pontisso et al, 1989)

Table 1.6 Examples of virus cellular receptors (Lentz, T.L., 1990)

RNA VIRUS	HOST CELL RECEPTOR	BINDING DOMAIN OF RECEPTOR	BINDING DOMAIN OF VAP
Picornaviridae			
Enterovirus			
Poliovirus	Member of the immunoglobulin superfamily (Mendelssohn et al , 1989)	Residues 33-137 (domain 1) (Koike et al, 1991)	Residues 95 - 105 of VP1 capsid protein (Murray et al, 1988)
Rhinovirus			
Human rhinovirus	Intercellular adhesion molecule - 1 (ICAM-1) (Greve et al., 1989; Staunton et al., 1989; Tomassini et al., 1989)	Residues 1-168 (Domain 1 and 2) (Staunton et al, 1990)	Residues of VP1 and VP3 major capsid proteins lining a canyon on the virus surface (Rossmann et al., 1985) Lys-103, Pro-155, His-220, Ser-223, of VP1 protein. (Colonno et al., 1988)
Cardiovirus			
Encephalomyocarditis virus	Sialoglycoproteins (Burness et al., 1981)		
Mengo virus			Residues of VP1 and VP3 major capsid proteins lining a pit on the virus surface (Luo et al., 1987)
Apthovirus			
Foot-and-Mouth Disease Virus	Integrins (Adhesion proteins) (Fox et al., 1989)		Residues of 145-147 (Arg, Gly, Asp) and 203-213 of VP1 protein (Fox et al., 1989) Residues 133-158 and C-terminal region of VP1 protein (Acharya et al., 1989)
Reoviridae			
Reovirus			
Reovirus 3	β-adrenergic receptor (Co et al., 1985) Sialoglycoproteins (Paul and Lee, 1987)		C-terminal portion of haemagglutinin (Bassel-Duby et al., 1985)
Togaviridae			
Alphavirus			
Semliki forest virus	Class I HLA and H-2 MHC molecules (Helenius et al., 1978)		
Lactate dehydrogenase - elevating virus	Class II 1a MHC molecule of macrophages (Inada and Mims, 1984)		

Table 1.6 Continued

RNA VIRUS	HOST CELL RECEPTOR	BINDING DOMAIN OF RECEPTOR	BINDING DOMAIN OF VAP
Orthomyxoviridae *Influenzavirus* Influenza virus	Sialyloligosaccharide (Paulson et al, 1979; Paulson, 1985)	Influenza A: NeuAcα2,6Galβ1,4GlcNAc NeuAcα2,3Galβ1,3GalNAc NeuAcα2,3Galβ1,4GlcNAc (Paulson et al., 1979; Carroll et al., 1981; Rogers and Paulson, 1983) Influenza C: 9-O-AcNeuAc (Rogers et al., 1986)	Residue 226 of haemagglutinin (HA) (Rogers et al., 1983) Tyr-98,Trp-153,His-183,Glu-190,Leu-194, and neighbouring pocket residues of HA (Wiley & Skehel, 1987) Tyr-98,Gly-134,Gly-135, Ser-136,Asn-137,Ala-138,Trp-153, Thr-155,His-183,Ser-186,Glu-190,Leu-194,Tyr-195,Leu-226,Ser-228 of HA (Weiss et al., 1988)
Paramyxoviridae *Paramyxovirus* Sendai virus	Sialyloligosaccharides (Paulson et al., 1979)	NeuAcα2,3Galβ1,3GalNAc (Paulson et al., 1979) NeuAcα2,8NeuAcα2,3Galβ 1,3GalNAc (Holmgren et al., 1980)	
Newcastle disease virus	Sialyloligosaccharides (Paulson et al., 1979)	NeuAcα2,3Galβ1,3GalNAc (Paulson et al., 1979)	
Rhabdoviridae *Vesiculovirus* Vesicular stomatitis virus	Phosphatidylserine (Schlegel et al., 1983; Mastromarino et al., 1987) Phosphatidylinositol (Mastromarino et al., 1987) GM3 ganglioside (Mastromarino et al., 1987)		
Lyssavirus Rabies virus	Acetylcholine receptor (Lentz et al., 1982) Sialylated gangliosides (Superti et al., 1986)	Residues 173-204 of acetylcholine receptor α subunit (Lentz et al., 1987)	Residues 151-238 of G protein (Lentz et al., 1984)

Table 1.6 continued

RNA VIRUS	HOST CELL RECEPTOR	BINDING DOMAIN OF RECEPTOR	BINDING DOMAIN OF VAP
Retroviridae *Oncovirinae* Human T cell leukaemia virus (HTLV - 1)	Class I HLA MHC molecule (Clarke et al., 1983) Interleukin 2 receptor (Lando et al., 1983; Kohtz et al., 1988)		Residues 246-253 of envelope gp (Kohtz et al., 1988)
Murine leukaemia virus	Lymphoma cell surface IgM (McGrath et al., 1987) 622 amino acid, hydrophobic protein of unknown function (Albritton et al., 1989)		
Radiation leukaemia virus	T - cell receptor - L3T4 molecule complex (O'Neill et al., 1987)		
Lentivirinae *Lentivirus* HIV-1	CD4 molecule of T lymphocyte (Dalgleish et al., 1984;Klatzman et al., 1984; McDougal et al., 1986) CD4 molecule interacting with class II HLA - DR MHC molecule (Mann et al., 1988)	Residues 48,50,51,121-123 of CD4 molecule (Clayton et al., 1988) Residues 37-53 of CD4 molecule (Jameson et al., 1988) Residues 37-83 of CD4 molecule (Landau et al., 1988) Residues 31,44,48,52,55,57, of CD4 molecule (Mizukami et al., 1988) Residues 42-49 of CD4 molecule (Peterson and Seed, 1988) Residues 41-55 of CD4 molecule (Arthos et al., 1989)	Residues 193-200 of gp120 (Pert et al., 1986) Residues 426-437 of gp120 (Lasky et al., 1987) Regions including residues 363,419,473,and 39 C-terminal residues (Kowalski et al., 1987) Residues 468-511 of gp120 (Linsley et al., 1988) Trp 432 (Cordonnier et al., 1989a)
HIV-2	CD4 molecule (Guyader, 1987)		
Simian imunodeficiency virus	CD4 molecule (Kannagi et al., 1985; Kornfeld et al., 1987; Hoxie et al., 1988)		
Coronaviridae	Carcinoembryonic antigen family (Ig superfamily) (Dveksler et al.,1991)		

Table 1.6 continued

Alternatively, a number of viruses bind, with considerable specificity, to cell surface molecules which are common to many cell types. Such viruses include orthomyxoviruses and paramyxoviruses, which bind sialic acid. Sialic acid is present on all mammalian cells, with the only variations being in the linkages between the sialic acid residues and adjacent sugars. As well as occurring on most mammalian cell types, sialic acid is often also the terminal sugar on oligosaccharides of glycoproteins and glycolipids, and consequently myxoviruses can bind to a wide variety of cells. Sialic acid has also been implicated in the binding of picornaviruses, papovaviruses, reoviruses and adenoviruses (Burness et al., 1981). Complex carbohydrate moities have also been associated with the binding of many other viruses (e.g. herpes simplex virus, which utilizes heparan sulphate as the initial cellular binding site).

Viruses that use common cell surface molecules as binding receptors, and as a result display a broad binding tropism, do not necessarily replicate in those cells but instead require intracellular determinants of replicative tropism.

Certain virus groups, such as Alphavirus, rhabdovirus, and vesicular somatitis virus (VSV) also appear to have a broad binding host range but have a far lower affinity for their receptors, and in some cases a far lower specificity. SFV, like several other viruses, appears to use more than one type of receptor molecule as a method of entry into cells. It is known to bind to the major histocompatability complex (MHC) class I on lymphoblastoid cells (Helenius et al., 1978), but is also known to bind to cells that do not bear these proteins (Oldstone et al., 1980). Several virus groups may share the same cellular receptor, as has been demonstrated by competition binding assays. On HeLa cells for example, four distinct binding sites are used by a number of non-enveloped viruses. Group 1 by HRV 2, 1A and 1B; group 2 by coxsackievirus type A21 and HRV types 3, 5,

14, 15, 39, 41, 51; group 3 by all 3 serotypes of poliovirus and group 4 by coxsackievirus B3, other coxsackieviruses and adenoviruses 2 and 5.

Two other common mechanisms have also been described for initial virus cell interaction; the use of coat proteins from other viruses, expressed on the surface of an infected cell as a cellular receptor, and the binding of virus to cells via an intermediary molecule.

In the first situation, a cell may be infected with a virus, such as influenza which expresses its HA on the cell surface. Since HA binds specifically and with great affinity to sialic acid, any virus bearing sialic acid as part of a glycoprotein on its surface (e.g. the envelope G protein of VSV) is able to utilize the influenza HA as a receptor to subsequently infect the cell (Fuller et al., 1985). Though this effect has only been shown *in vitro*, these 'secondary infections' may also occur *in vivo*.

Use of an intermediary molecule, as opposed to direct interaction of virus with cell, is not uncommon, with some of the best examples occuring via antibody molecules. Antiviral antibodies enhance the binding of flaviviruses, myxoviruses and lentiviruses, where antibody cross-links the virus and the cell via the cells F_c receptors (McGuire et al., 1986; Peiris and Porterfield, 1979; Peiris et al., 1981; Webster and Askonas, 1980) Hepatitis B virus is believed to bind to hepatocytes via polyalbumin and polymeric albumin receptors (Thung and Gerber, 1984). The continued role of the cellular receptor after binding and endocytosis varies from virus to virus. The receptors for influenza virus, alphavirus and rhabdovirus are not required for fusion to target cell membranes, whereas the receptor for poliovirus may be needed to ensure the correct conformational changes in the virus in order to facilitate entry into the cell from the endosome. The ability of a cell membrane molecule to act as a receptor for a virus is not simply determined by whether the sequence for that protein is encoded in the target cell genome. For instance, RNA coding for the poliovirus receptor has

been detected in many different cell types, even though the virus is known not to infect these cells. It is possible that cell specific post-translational modifications are required for the protein to be expressed on the cell membrane, or that the presence of additional proteins is needed for successful virus binding and internalization. Equally, Influenza virus utilizes sialic acid as a cell binding site, but does not infect all cells bearing that moiety, even if the virus subsequently enters the cell by endocytosis and membrane fusion.

In these cases it is necessary for a more complex cell membrane mechanisms (as seen with herpes simplex virus) or an intracellular mechanism such as cellular polymerase specificity to determine cellular tropism.

1.2.5 Clinical Implications of Virus - Cellular Receptor Interaction

One important feature of a the interaction between a virus and its cellular receptor is that in order to maintain the ability to bind to a particular cell type, the virus cannot mutate its cell binding site to any degree. As a consequence, either the cell binding site on the virus, or the cellular receptor on the target cell can be used as a target for anti-viral therapy, with little or no danger of major changes to their structure. This is of particular relevance in the case of viruses such as HIV and Influenza, which evade the host's immune response by frequently altering their envelope glycoproteins. Some viruses have evolved "canyons" on their surfaces, at the bottom of which lies the cell binding site. The canyon is of such a conformation that antibodies to this area are unable to bind to the cellular binding site. This type of capsid conformation has been described in Rhinovirus (Rossmann et al., 1985), Mengo virus (Luo et al., 1987) and on the haemagglutination glycoprotein of the Influenza virus (Weis et al., 1988), where a three-dimensional structure of HA complexed with sialyllactose shows the acid filling the pocket. The role of antibodies directed to the rims of these canyons, and subsequent steric interference with binding, has yet to be determined for

many viruses. In the case of Influenza virus it has been shown that neutralizing monomeric IgG and IgA do not inhibit binding, whereas larger polymeric IgA and IgM do reduce binding (Taylor and Dimmock, 1985a, b). The neutralization site for Rhinovirus 14 is located on protrusions from the virus surface (Sherry et al., 1986) as is the neutralization site for Foot-and Mouth-Disease-Virus (FMDV) which does not possess a canyon at all (Acharya et al., 1989). In this instance the cell binding motif of arginine - glycine - aspartic acid is situated in a hypervariable immunodominant region (Fox et al., 1989) which may suggest that a cellular binding site on the virus may evade antibody neutralization by being too small (Colman et al., 1983; Harrison, 1989)

Since viruses often utilize cell membrane bound molecules which have a normal physiological function, it is not surprising that the sequence of cell binding proteins on viruses often show homology with natural ligands (see table 1.7). As a result, binding of viruses can affect the target cell physiologically, or even subvert the immune response to it by causing autoimmune disease to tissues carrying that particular characteristic (Oldstone, 1987). All of these events are generally termed molecular mimicry. (Damian, 1987). The physiological effects of a virus binding to one of its receptors may, in some cases, be so drastic that they manifest themselves as symptoms of the disease, particularly if the virus sheds an excess of its binding protein. The vaccinia virus encoded VGF protein, for example has been shown to stimulate tyrosine kinase, as a result of its similarity to epidermal growth factor (King et al., 1986); and the receptor-mediated leukaemogenesis hypothesis proposes that murine leukaemia viruses induce lymphocyte proliferation by binding to antigen - specific receptors complementary to virus envelope gene products (McGrath et al., 1987). The psychological symptoms experienced by rabies victims may be accounted for by the rabies virus

Adenovirus glycoprotein (102-116) Immunoglobulin M κ chain, human (83-97)	DITMYMSKQYKLWPP DIATYYCQQYNNWPP
Epstein-Barr Virus gp350 (372-378)(21-29) Complement C3d fragment (1006-1012)(1221-1231)	TPSGCEN EDPG--FFNVE TPSGCGE EDPGKQLYNVE
Human Cytomegalovirus H301 gene product (261-286) Class I MHC molecule α-3 domain, human (consensus)	DGTFHQ--GCYV-AIFCNQNYTCRVTH DGTFQKWAAVVV-PSG-EQRYTCHVQH
HIV-1 gp120 (193-200) Vasoactive Intestinal polypeptide (4-11)	ASTTTNYT AVFTDNYT
HIV-1 gp120 (61-88) Immunoglobulin γ heavy chain, human (84-111)	CASDAKAYDTEVHKVWATHACVPTDPNP CNVDHKPSNTKVDKTVERKCCVECPPCP
HIV-1 gp120 (245-273) Neuroleukin (410-438)	VQCTHGIRPVVSTQLLLNGSLAEEEVVIR VQTQHPIRKGLHHKILLANFLAQTEALMR
HTLV-1 gp (246-253) Interleukin 2, human (20-27)	SVPSSSST SAPTSSST
Rabies virus gp (189-199) *Naja naja naja* (India) toxin b (30-40)	CDIFTNSRGKR CDGFCSSRGKR
Vaccinia virus VGF protein (69-80) Epidermal growth factor, human (31-42)	CRCSHGYTGIRC CNCVVGYIGERC

Table 1.7 Examples of similarities between virus attachment proteins (VAP) and their mimics. (reproduced form Lentz, T.L. 1990)

glycoproteins, which can compete for binding to acetylcholine receptors (Donnelly - Roberts and Lentz, 1989).

There are theoretically two groups of compounds which should be able to block virus binding to its target cell; the first are natural or synthetic molecules which mimick the cell binding site of the virus. The other group are compounds which mimick the cellular binding site on the cell surface. Examples of both of these groups have been shown to be effective.

(i) Agents Mimicking the Virus's Attachment Protein ('VAP mimics')

Antibodies to the CD4 molecule of the HIV virus target cell can be inhibitory to virus binding (Landau et al., 1988; Mizukami et al., 1988), as can antibodies raised against monoclonal antibodies to the HA of reovirus (anti idiotypic antibodies) (Kauffman et al., 1983). These antibodies have particular importance since they can elicit an immune response to a virus without exposure to the virus itself. Natural ligands, physiological or not, can inhibit binding of a virus; such as α-Bungarotoxin for Rabies virus (Lentz et al., 1982) or epidermal growth factor in the case of Vaccinia virus (Eppstein et al., 1985). However, these types of compounds have little therapeutic use since they also exert toxic effects. The last type of compound able to mimic virus attachment protein are synthetic peptides based on known sequences.

(ii) Agents Mimicking the Cellular Binding Site

Essentially, these compounds mirror the actions of the 'VAP mimics'. As has been described in section 1.2, antibodies to the coat protein of the virus will compete with the cellular binding site and may, in some cases, neutralize the virus. These antibodies can be raised to whole virions, purified virus structural proteins, or synthetic peptides known to represent immunogenic regions of the virus. These methods have been used successfully with numerous viruses, including hepatitis B virus (Itoh et al., 1986), the VP1 protein of FMDV

(DiMarchi et al., 1986), HIV gp120 (Lasky et al., 1987; Dowbenko et al., 1988; Sun et al., 1989) and human rhinovirus (McCray and Werner, 1987). Such neutralizing antibodies do not necessarily have to be directed to the binding site itself, but to a site sufficiently close to inhibit binding by steric hindrance. Anti-idiotypic antibodies against an anti-CD4antibody mimic the CD4 molecule and bind the HIV glycoprotein, partially neutralizing the virus. Again, synthetic chemicals can be assembled which contain the virus binding site, as with Influenza virus where compounds such as NeuAcαCH_2, NeuAcαC_7H_7 as well as sialyloligosaccharides containing NeuAcα2,6GlcNAc or NeuAcα2,6Gal (depending on the strain used) inhibit virus adsorption to erythrocyte membranes (Pritchett et al, 1987)

1.2.6 Methods of Characterization and Purification of Virus Cellular Receptors

(i) Anti-Receptor Monoclonal Antibodies

One of the most popular methods for the purification of virus cellular receptors is the raising of a monoclonal antibody to it, and to use this to purify the receptor, by some form of immunoaffinity chromatography. This type of monoclonal antibody can be isolated by screening for its ability to block infection, since in many cases (but not all, see Herpes Simplex Virus) the only molecule relevant to virus infection, on the surface of a permissive cell, is the cellular receptor. Generally, mice are immunized with whole cells, as they are more antigenic than sub - cellular fractions. Having established that the animal is producing antibodies capable of blocking infection (by pretreating a cell monolayer with a tail vein bleed prior to infection, and subsequently identifying plaque reduction) the spleen cells can be immortalized by fusion with mouse myeloma cells, using methods which have been described previously (Kohler and Milstein, 1975). The resulting hybridomas can be tested to find a monoclone which will inhibit infection, as has

been successfully accomplished with the major group Rhinoviruses (Colonno et al., 1986).

If this highly labour - intensive task is successful, then the relevance of the monoclone can be confirmed by inhibiting the binding of radiolabelled virus to a permissive cell line, as opposed to simply inhibiting infection. If this too proves successful, the monoclonal antibody can then be used to immunoaffinity purify the receptor for detailed structural analysis, such as glycosylation status and N - terminal sequencing.

Having isolated an anti-receptor monoclonal antibody, it is also useful to test for its ability to block the binding of other viruses, since the sharing of receptors is known to occur. Such a comparison has been performed with the anti - receptor antibody for the major group rhinoviruses, where it was discovered that the same antibody also inhibited the binding of Coxsackievirus A21, though it did not inhibit the binding of other Coxsackieviruses or Poliovirus.

Molecular Cloning

It has been established that suceptibility to infection by a virus can be conferred upon a cell line by transforming that cell line with DNA from permissive cells. This was most notably achieved by Mendelssohn et al.(1986) with the transformation of non-permissive L-cells with human DNA. Mendelssohn et al., (1989) subsequently used these techniques to clone the poliovirus cellular receptor directly. The group repeated the earlier work, by transforming mouse L cells with DNA from HeLa cells to create CM-1 cells, and testing for poliovirus antigen production by standard plaque assay. In order to isolate the virus cellular receptor, L cells were transformed with DNA from the CM-1cells, and the presence of human sequences containing the receptor established by plaque assay, restriction digests and the use of a DNA probe for Alu repeats (a characteristically human sequence). As a result, it was established that the poliovirus receptor has

an Alu repeat associated with it in a 10kb fragment. A genomic library was constructed from one secondary transformant containing this fragment and was screened for Alu repeat sequences. Three bacteriophage recombinants were thus isolated. One recombinant contained mouse sequences and the other two contained a 1kb BamH1 fragment, which was present in all secondary transformants, and hybridized with DNA from HeLa cells and SY5Y neuroblastoma cells (both permissive) but not L cells (not permissive). This 1 kb fragment was then used to probe HeLa cell cDNA libraries for the whole receptor. By this method, an open reading frame was cloned, which when transfected into non - permissive cells, rendered them liable to infection with poliovirus.

Virus Overlay Protein Blot Assay (VOPBA)

This approach was used for the estimation of the molecular weight of the putative cellular receptors for Visna Virus (Crane et al., 1991; Dalziel et al., 1991), Human Cytomegalovirus (Adlish et al., 1990) and Lymphocytic Choriomenigitis Virus (Borrow and Oldstone, 1991).

Essentially, whole cells or membrane preparations from cells known to be permissive for the virus under study, are subjected to SDS - PAGE and western blotted onto nitrocellulose. The blot is then incubated with labelled virus and the position where the virus has bound is visualized by an apppropriate method. Alternatively, the virus may be indirectly detected by a labelled, virus - specific polyclonal or monoclonal antibody. The molecular weight of the virus - binding sites on the cell surface can then be calculated. This method can be extended to investigate which cell lines the virus may bind to, which viruses may share the same binding sites, and what agents inhibit virus binding. The main problem with this method is that the virus binding site, in many cases, will be disrupted during SDS - PAGE, and so the virus - receptor interaction must be able to withstand this to remain active.

Anti-idiotypic Antibodies

Anti-idiotypic antibodies are antibodies raised to the F_{AB} portion of a monoclonal antibody raised to regions of the virus surface. As such, it is possible that they may be able to mimic the cell binding sites on the virus surface, and therefore may be used as a probe for, and subsequently immunoprecipitate, the virus's cellular receptor. In the case of Sindbis Virus (Wang, K.-S. et al., 1991) it was found that such an antibody could partially inhibit virus infection of chicken cells, but would not inhibit the infection of BHK cells, suggesting that Sindbis Virus is able to utilize more than one type of cell surface protein as a means of entry. Although a bank of anti - virus monoclonal antibodies can be used as immunogens, it is clearly easier to make use of this technique if an antibody is known to neutralize through the virus's cell binding protein.

Interference with Virus Binding

It has also been possible to discover the nature of the cellular receptor either by incubating the virus with compounds which mimic the cellular receptor, or by treating the surface of the target cell with various enzymes.

The best example of this is the inhibition of influenza virus - induced haemagglutination, by first treating the red blood cell surface with neuraminidase to remove the sialic acid residue of oligosaccharides.

This proved that sialic acid can play an important part in virus binding and entry into the cell. Use of neuraminidase has been extended to other viruses such as bovine coronavirus, encephalomyocarditis virus and picornaviruses.

The binding site of Herpes Simplex Virus has been shown to involve cell surface heparan sulphate, by interfering with infection using heparin or enzymes which very specifically destroy heparin sulphate, such as heparinase and heparitinase (WuDunn and Spear, 1989).

Copurification of Virus and its Cellular Receptor

It is possible to use the virus itself as an affinity ligand in an attempt to purify the cell surface receptor. Mapoles et al. (1985) isolated a tightly bound virus - receptor complex by incubating Coxsackievirus B3 with HeLa cells, and then solubilizing the cells with sodium deoxycholate and Triton X-100. The VRC was then purified 30,000 fold by differential and sucrose gradient ultracentrifugation. Iodination of the VRC, and SDS-PAGE analysis allowed the group to estimate the molecular weight of the binding protein. This approach is the only direct method of purifying the whole receptor in one step, and provided yields are good, allows a very effected way of gaining detailed information about the receptor.

1.3 AIMS OF THE PROJECT

At the commencement of the project, the information available regarding the distribution and structure of the cellular receptor for hepatitis A virus was extremely limited, relying mainly on clinical data and comparisons with other picornaviruses.

In particular, one important question remained unanswered; does hepatitis A infect gut (and other) cells in order to finally infect (and reinfect) the liver? (see Fig.1.8) It was known that poliovirus can infect gut cells during its passage from the gut to the neuronal cells, so it was suggested that this must be the case for hepatitis A, though evidence had yet to be obtained. One way of answering this question would be to establish a method of screening certain cell types for their ability to support virus replication. This has been achieved for other viruses by confirming the presence or absence of certain cell surface molecules which act as their cell binding sites.

Therefore, the first aim of the project was to investigate the possibility of raising an antibody to the surface of FRhK-4 cells which would block the binding of the

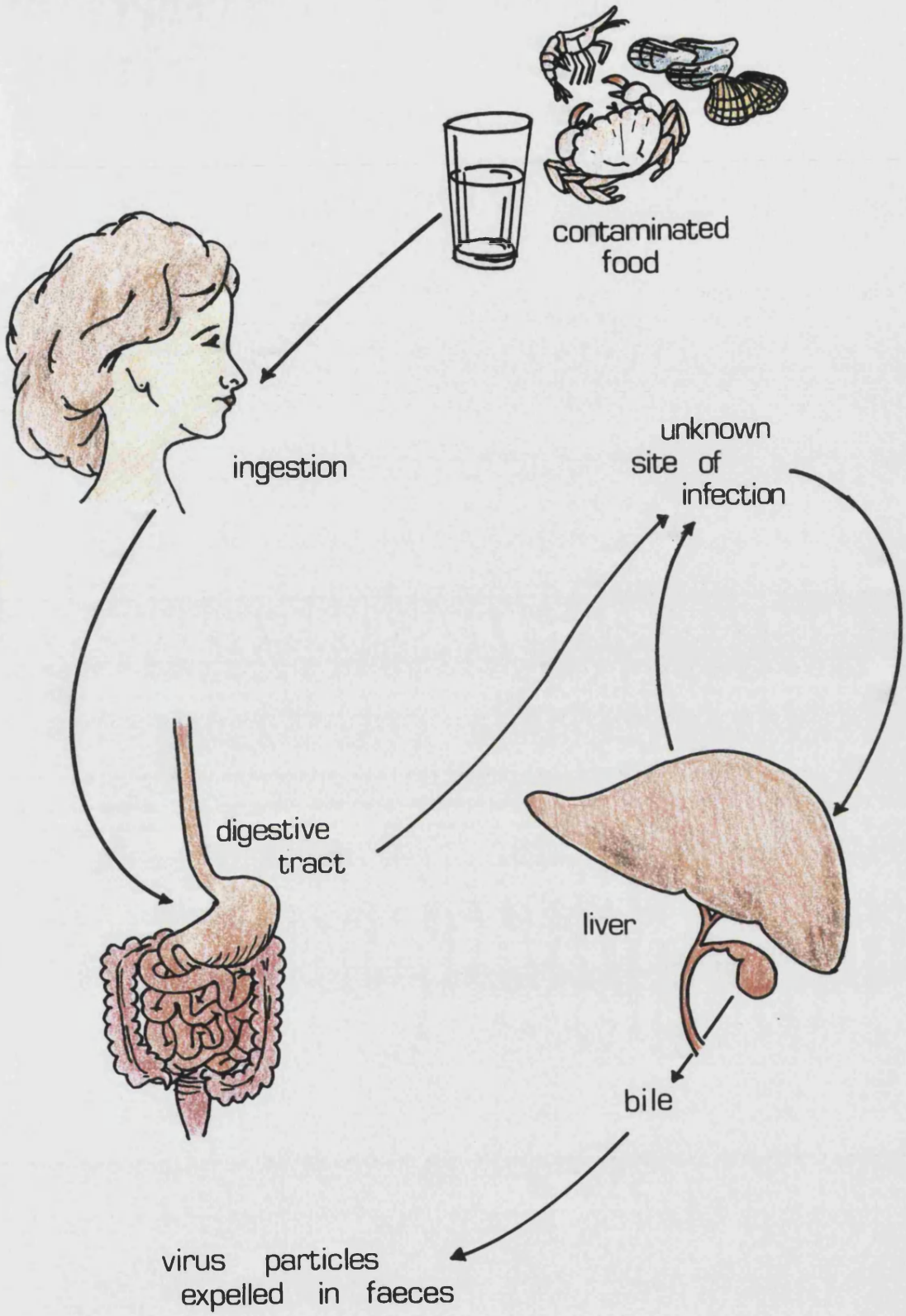


Fig. 1.8 Pathological mechanism of hepatitis A.

virus. This was likely to be an extremely time consuming undertaking, since it would be impossible to screen initially for inhibition of infection (typical HAV replication times in cell culture are between 7 and 14 days). As a result, the only option was to develop a cell binding assay and to use this in a cell protection assay (HAV versus monoclonal antibody competition binding assay).

In keeping with the general approach to monoclonal antibody isolation, some attempt would also be made to purify or partially purify the cellular receptor by co-isolating it with purified HAV. Two approaches could be investigated; a virus- receptor complex could be purified by the same method as for standard virus purification, or the cellular receptor could be localized on SDS-PAGE by virus - overlay protein blot assay. Both preparations could then be used as immunogens for antibody production. These two methods would also yield information as to the molecular weight of the receptor and indirectly, its composition.

At the time, the inhibition of virus binding by a polyclonal antibody to the surface of FRhK-4 cells had been shown, by other workers, to be unattainable (Anderson, D.A. personnal communication) and so other methods would be used to gather general information about the receptor and its distribution.

Though not strictly applicable to the clinical situation, a study of virus binding to a range of cells was considered relevant, to try to establish discrete binding between permissive and non-permissive cells. It was intended that this would be extended to look at some features of the kinetics of virus-cell interaction and also the number of receptor copies per cell. The binding assay would also be used to investigate which treatments of the cell surface would repress binding. This information could then be interpreted to gain some basic information about the composition of the receptor.

The neutralization of HAV by monoclonal and polyclonal antibodies was also an area which lacked information. The most relevant piece of information was the

observation that there is only one wild type serotype of HAV known. Therefore it was proposed that to escape neutralization by polyclonal antibody may well prove fatal to the virus. Three HAV - specific monoclonal antibodies were available, two shown to neutralize HAV and one not. These monoclonal antibodies would be used to investigate the possibility that the portion of the virus capsid which binds to the cellular receptor may also represent an epitope for neutralizing antibodies.

2. MATERIALS AND METHODS

Unless otherwise stated all tissue culture disposables, media and supplements were obtained from Gibco, Flow and Sterilin and all reagents were analytical grade obtained from Sigma or BDH.

2.1 CELLS

Foetal Rhesus monkey kidney cells (FRhK-4) were provided by Dr. B. Flehmig, Hygiene - Institute, Department of Medical Virology, Tübingen, Germany and grown at 37°C in Dulbecco's modification of Eagles medium. Chinese hamster ovary cells (CHO) were grown at 37°C in Ham's F12 medium and WRC were grown at 37°C in 199 medium. All media were supplemented with 2mM glutamine, 10% foetal calf serum (FCS), 100µg/ml streptomycin and 100U/ml penicillin. Cells were split weekly after treatment with 0.5% trypsin and maintained in 75 cm^2 flasks.

2.2 VIRUS

The HM175 strain of HAV was originally recovered from the faeces of a naturally infected human in Australia. It was provided by Dr. John Wood, National Institute of Biological Standards and Controls, Potters Bar, Herts having been passaged 6 times in marmoset livers, 10 times in AGMK cells and twice in BS-C-1 cells. (Daemer et al, 1981; Coronas et al, 1989) To produce HAV HM175 seed, a confluent monolayer of FRhK-4 cells was inoculated with 800 pfu/ml of cell associated virus seed in PBS for 90 min. at 35°C. The inoculum was then removed and replaced with DMEM maintenance medium (DMEM supplemented with 1% FCS, 2mM glutamine, 100µg/ml streptomycin, 100U/ml penicillin) for 10 days at 35°C after which the medium was removed and clarified by centrifugation at 15.000g for 20 min. and stored at -70°C until required (supernatant (s/n) virus stock). The infected monolayer

was lysed by three cycles of freeze / thawing in dry ice and clarified by centrifugation at 15,000g for 20 min. and then stored at -70°C until required (cell associated (c/a) virus stock 8 x 10^4 pfu/ml). Cell associated virus stocks were used in subsequent passaging of the virus at a multiplicity of infection (m.o.i.) of 4 x 10^{-4}.

2.3 SERA AND ANTIBODIES

Pooled IgG fractions of human convalescent sera was provided by Dr. Hemda Garelick, London School of Hygiene and Tropical Medicine, London, WC1. Pooled convalescent sera was provided by Dr. Diane Westmoreland, Public Health Laboratory Service, Bath, Avon. An HAV specific, non - neutralizing mouse monoclonal antibody, 14H / LSHTM was provided by Dr. Hemda Garelick, London School of Hygiene and Tropical Medicine, London, WC1. Two HAV specific neutralizing mouse IgG monoclonal antibodies, 813 and 10.09 were provided by Clonatec - Biosoft departément, 60, rue de Wattignes, 75580, Paris cedex 12.(Crevat et al, 1990; Ping and Lemon 1992)

2.4 PROTEIN ESTIMATION

A series of bovine serum albumen protein concentration standards were made up in PBS from a stock solution of 0.5 mg/ml to give final concentrations of 2, 4, 10, 20 and 40 μg/ml in 100μl in microtitre plate wells.
The working detection solution was made up as follows. Three solutions were made up, each of which are stable at room temperature. Microreagent A (MA) (4g sodium carbonate monohydrate, 0.8 g sodium hydroxide, 0.8 g sodium tartrate in 50 ml ddH_20 [pH 11.25, with sodium tartrate] ; microreagent B (2 g bicinchoic acid (Pierce) in 50 ml double distilled H_20 (ddH_2O)) and microreagent C (40μl 4% copper (II) sulphate and 960 μl MB). The final working solution was 1 volume of MA added to 1 volume MC which was prepared on the day of use.100μl of the working solution was added to the

protein standards and to the appropriate dilutions in 100µl of the protein test samples and incubated at 60°C for 1 hr. The result was then be read on a microtitre plate reader at 562nm.

2.5 SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS.

Electrophoresis in the presence of SDS was performed using a method based upon that of Laemmli (1970).

A 12.5% polyacrylamide slab gel was poured and overlaid with water saturated butanol. Once it had set the water/butanol was removed and a 4% polyacrylamide stacking gel poured on top, wells were formed by placing a comb in the top. 0.248M Tris - HCl [pH 8.7] and 0.345M Tris - HCl [pH 6.8] buffers were used for the resolving and stacking gel respectively. In both cases polymerization was achieved by the use of ammonium persulphate and N,N,N',N - tetramethylethylenediamine (TEMED).

The protein samples were mixed with sample buffer (12.5% v/v 0.5M Tris - HCl [pH 6.8], 20% v/v glycerol, 2% w/v SDS, 5% v/v β - mercaptoethanol and 0.0025% w/v bromophenol blue) and boiled for 5 min prior to loading. Molecular weight markers (Pharmacia LKB) were made up according to manufacturers instructions and also boiled prior to loading. The gel was run at 20 - 30 mA (30 mins.) for the stacking gel and increased to 40-50mA following the interface with the resolving gel (4hrs).

2.6 COOMASSIE STAIN OF POLYACRYLAMIDE GELS

Following electrophoresis the gel was stained for 1 hr at room temperature in coomassie blue stain (0.25% w/v coomassie blue stain in fixing solution (30% methanol, 10% acetic acid, 60% ddH_2O)) and then destained in several changes of fix until the background colour was removed.

2.7 SILVER STAIN OF POLYACRYLAMIDE GELS.

Following polyacrylamide gel electrophoresis, the gel was prefixed in a 50% methanol, 10% acetic acid solution for 30 min and then a 5% methanol, 7% acetic acid solution, also for 30 min. Without rinsing the gel was then fixed for 30 min in 10% gluteraldehyde and washed in a large volume of ddH_2O overnight followed by a quick wash in ddH_2O the next day. Following a 30 min soak in 5μg/ml dithiothreitol the gel was immersed in a 0.1% solution of silver nitrate for 120 min. The gel was then briefly washed once in ddH_2O and twice in developer (50μl 37% formaldehyde in 100 ml 3% sodium carbonate). The protein bands were visualized by bathing the gel in developer until the desired level of staining had been attained. Staining was stopped by adding 5 ml 2.3M citric acid to the developer and shaking for 10 min. The gel was washed three times in ddH_2O and once in 0.03% sodium carbonate before being sealed in a plastic bag.

2.8 PROTEIN RADIO - IODINATIONS.

10μg of protein solution in 25μl 0.5 M sodium phosphate [pH 7.5] (0.5M Na_2HPO_4 adjusted to the correct pH with 0.5 M NaH_2PO_4) was added to 0.5 mCi $Na^{125}I$ in a 3ml stoppered plastic test tube and stirred with a small magnetic flea. The iodination reaction was started by the addition of 25μl 0.2 mg/ml chloramine T in sodium phosphate buffer and allowed to proceed for exactly 60 sec at room temperature with stirring. The reaction was terminated with the addition of stop buffer (0.24 mg/ml sodium metabisulphite, 10 mg/ml tyrosine, 10% glycerol and 0.1% xylene cyanol in PBS). The iodinated protein was separated from the ^{125}Iodotyrosine on an appropriate size exclusion column, in this case a 10 ml Sephadex G50 (Pharmacia LKB) swelled and blocked in 1% BSA in PBS followed by equilibration in 0.5M sodium phosphate. 0.5 ml fractions were collected and the position of the iodinated

protein established on an LKB gamma counter. All manipulations involving $Na^{125}I$ took place in an approved fume hood.(Greenwood and Hunter,1963)

2.9 QUANTITATION OF HAV - SPECIFIC ANTIBODIES BY DIRECT ENZYME-LINKED IMMUNOSORBENT ASSAY.

Three strips of eight microtitre plate wells were coated with a 1/1000 dilution of monoclonal antibody (mAb) 813 (0.6μg/ml), a 1/1000 dilution of mAb 10.09 (unknown concentration) and a 1/1000 dilution of mAb LSHTM/14H (unknown concentration) respectively, in bicarbonate buffer (0.16 % Na_2CO_3, 0.30% $NaHCO_3$, [pH9.6]) for 2 hr at 37°C, followed by an incubation for 2 hr at 37°C in blocking buffer (0.3% Tween 20, 1% dried milk powder in PBS) to occupy any vacant non - specific binding sites on the plastic. Doubling dilutions of the detecting , horse - radish peroxidase (HRPO) conjugated sheep anti - mouse IgG antibody (Amersham International, Amersham, Bucks, UK.) between 1/100 and 1/12800 were prepared in blocking buffer and added to each well such that each monoclonal antibody was exposed to each dilution of detecting, conjugated, monoclonal antibody. The wells were incubated with the detecting antibody for 2 hr at 37°C. The result was visualized with 100 μl 1% tetraaminobenzidine (in DMSO) in 10 ml of a 1/20 dilution of a citrate/acetate stock buffer (68 g sodium acetate, 3.15 g sodium citrate in 500 ml ddH_2O [pH 6.0]) containing 2μl H_2O_2. The reaction was stopped by the addition of 50μl 1.84M H_2SO_4 and the results read at 450 nm in a microtitre plate reader.

The results were obtained by the construction of a standard curve using values obtained for the known antibody concentration (813, 0.6mg.ml) (fig.2.1).

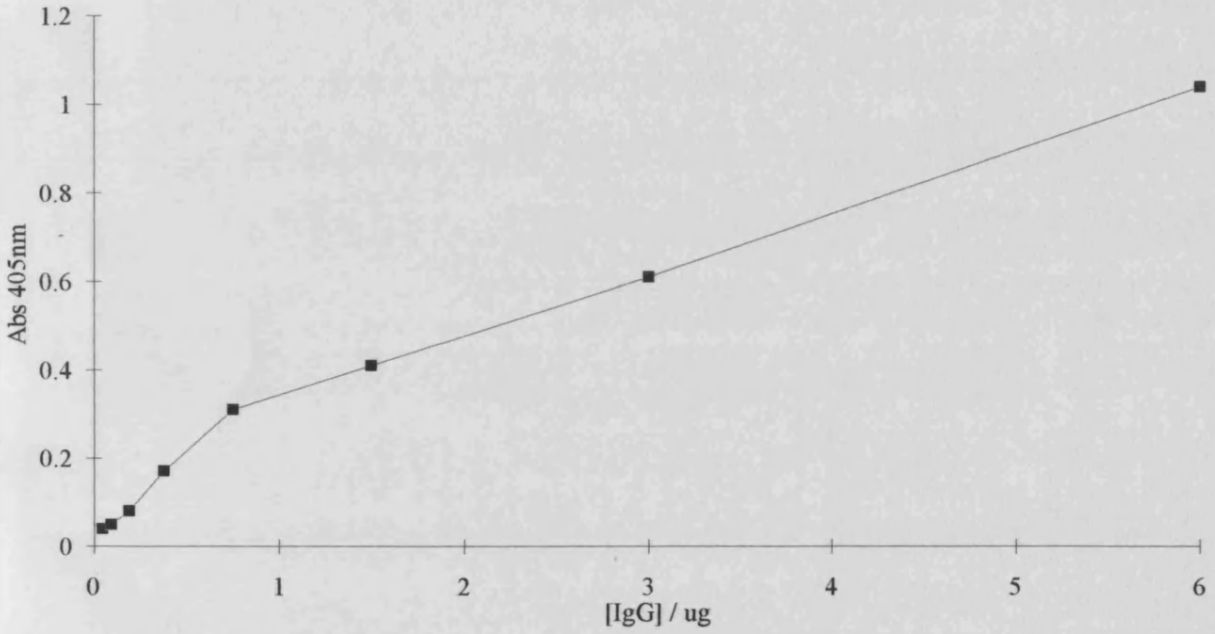


Fig. 2.1 Standard curve of [IgG] against absorbance for direct ELISA quantitation of anti - HAV monoclonal antibodies.

2.10 COMPETITION RADIOIMMUNOASSAY BETWEEN HAV-SPECIFIC ANTIBODIES

Microtitre plate wells were coated with a 1/1000 dilution of mAb 10.09 (0.22μg/ml) in carbonate coating buffer for 2 hr at 37°C, and blocked for 2 hr at 37° C in blocking buffer. A saturating dilution of HAV was then added to the wells, again in blocking buffer, and incubated at 37°C for 2 hr, followed by an incubation at 37°C for 2 hr with each competing monoclonal antibody, 10.09, 813 and LSHTM/14H at 0.22 μg/ml. The wells were then incubated with 1 x 10^6 cpm of [^{125}I] - mAb 10.09 for 2 hr at 37°C in blocking buffer. The wells were washed three times in blocking buffer between each incubation. To establish competition binding between the three monoclonal antibodies, the wells were cut away from each other, placed in LP4 tubes and counted on an LKB gamma counter.

2.11 HAV DETECTION BY SANDWICH, ENZYME-LINKED IMMUNOSORBENT ASSAY.

HAV specific mouse monoclonal antibody LSHTM/14H was diluted in bicarbonate buffer and used to coat polystyrene wells (0.33μg/ml) overnight at room temperature, and then blocked in blocking buffer for 2 hr at 37°C. Test samples of HAV in blocking buffer were loaded into the wells and incubated at 37°C for 2hr. An IgG fraction of pooled convalescent sera (0.88 μg/ml) was added to each well and incubated for 2 hr at 37°C, followed by a 1/1000 dilution of mouse anti-human monoclonal antibody, conjugated to alkaline phosphatase also at 37°C for 2hr in blocking buffer. Three washes in blocking buffer took place between each incubation. To develop the assay 100μl of carbonate buffer (0.16% Na_2CO_3, 0.30% $NaHCO_3$, [pH9.6]) containing 1.6 mg/ml p-nitrophenyl phosphate was added to each well. When the colour had

developed sufficiently, the absorbance was measured on a microtitre plate reader at 405nm.

2.12 AMMONIUM SULPHATE PRECIPITATION OF IgG.

FRhK - 4 specific mouse polyclonal serum was centrifuged at 3000g for 30 min to remove any large aggregates and sealed in activated dialysis tubing. The serum was dialysed overnight at 4°C against a large volume of 30% ammonium sulphate [pH 7.4] to slowly precipitate large protein aggregates which were then removed by centrifugation at 3000g for 30 min. The serum, in 30% ammonium sulphate was dialysed overnight at 4°C against a large volume of 50% ammonium sulphate to precipitate only the IgG fraction of the mouse serum. IgG was recovered in 0.5 volumes of PBS and dialysed against a large volume of PBS to remove any remaining ammonium sulphate. The resulting solution was centrifuged once more to remove debris, tested for purity on SDS - PAGE and stored at -70°C in 50% glycerol.

2.13 HAV PLAQUE ASSAY

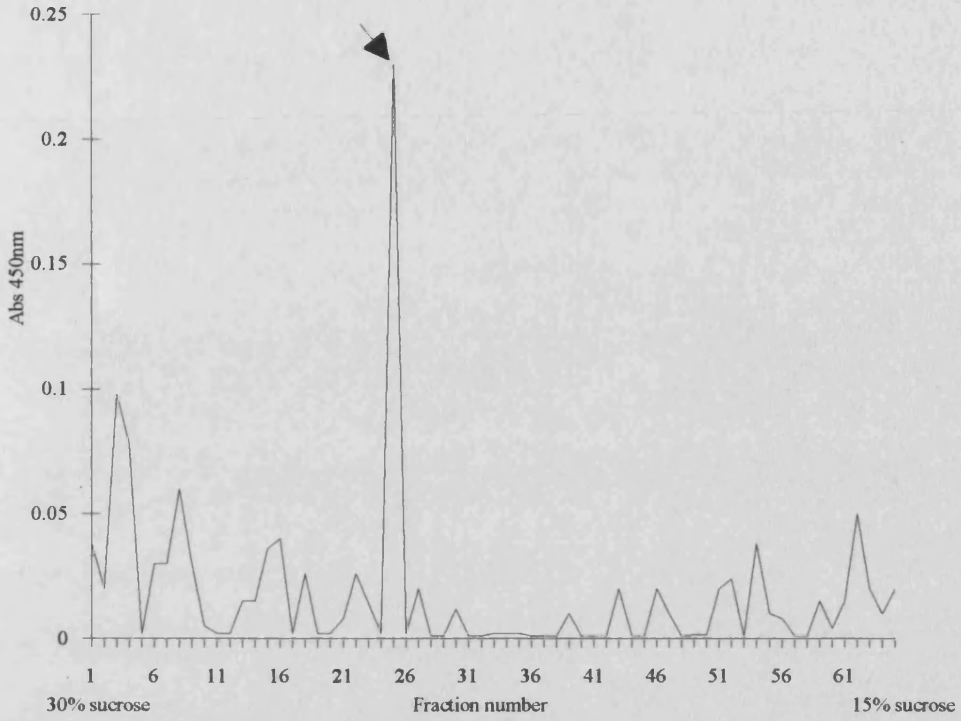
Confluent monolayers of FRhK- 4 cells were prepared in 10 x 60mm petri dishes. The cells were infected with serial dilutions of an HAV sample between 10^{-2} and 10^{-8} in PBS at 35°C for 90 min. The inocula were removed and the cells overlaid with DMEM maintenance medium containing 0.5% Seaplaque® low melting point agarose (FMC BioProducts, Rockland, ME, USA). After 10 days 4% formalin was laid onto the overlay and left to fix the cells for 1 hr at room temperature. Following gentle removal of the overlay, the plaques were visualized with a brief staining in 0.2% crystal violet.

2.14 HAV PURIFICATION.

FRhK-4 cells were infected with 8 x 10^2 pfu/ml of the HM175 strain of HAV seed and maintained at 35°C in DMEM maintenance medium. After 10 days the supernatant was pooled and centrifuged at 15,000 x g for 20 min to remove cellular debris. The infected cell monolayer was scraped into a small volume of lysis buffer (10 mM Tris-HCl, 10mM NaCl, 1.5 mM $MgCl_2$, 1% Nonidet ® P40, [pH7.5]) and incubated on ice for 10 mins followed by three rounds of freeze / thawing in a dry ice / 95% ethanol bath. The cell lysate was then centrifuged at 15,000g for 20 min to remove cell debris and the supernatant added to the clarified medium. This crude viral preparation was pelleted through a 20% sucrose cushion in 0.1% Sarkosyl® in TNE buffer (10mM Tris-HCl, 150mM NaCl, 1.0mM EDTA) for 20 hr at 100,000 x g on a Beckman SW27 ultracentrifuge rotor. The viral pellet was then slowly resuspended in 0.5% Sarkosyl® in TNE buffer and left overnight at 4°C. The virus suspension was then layered onto a 40 ml linear 15 - 30% sucrose gradient prepared in TNE buffer containing 0.5% Sarkosyl® and at 86,500 x g on a Beckman SW27 ultracentrifuge rotor for 4hr at 4°C. 0.5 ml fractions from the gradient were collected and tested for the presence of viral antigen using a sandwich ELISA (see 2.4). The major viral peaks (see fig. 2.2) were pooled, pelleted at 250,000g in a Beckman SW50 ultracentrifuge rotor for 2 hr in TNE buffer and resuspended in an appropriate buffer. (Hughes et al, 1984)

2.15 CELL BINDING ASSAYS

Cell monolayers were grown to confluence in 24 - well tissue culture plates in the appropriate growth medium. The cells were washed very gently three times with PBS and then incubated with a relevant dilution of [^{125}I]- HAV in binding buffer (the appropriate cell medium without FCS [1.8 mM Ca^{++}]). at 4°C for 2 hr. After the incubation period the [^{125}I]- HAV was removed but retained in 4



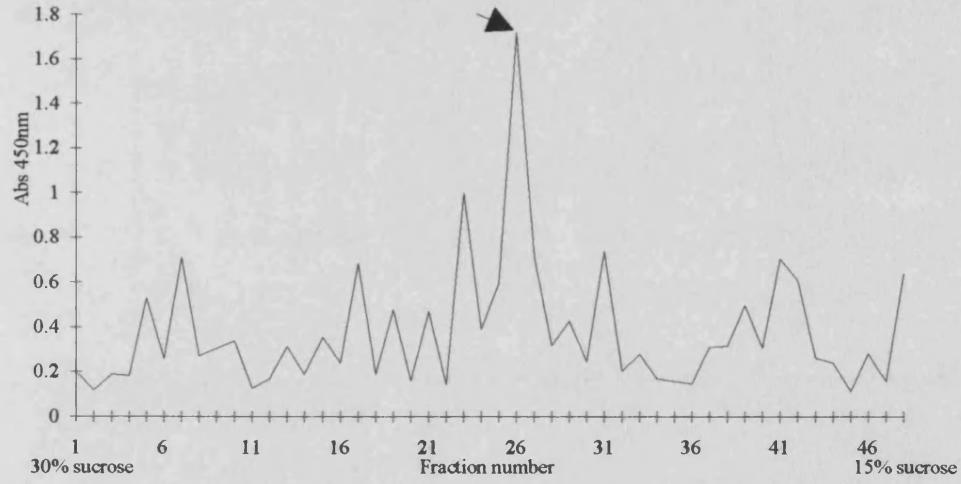


Fig.2.2 Sample sucrose gradients from the purification of HAV. HAV antigen detected by sandwich enzyme linked immunosorbent assay (see 2.11). The peaks highlighted were kept and used for subsequent assays. Heavier and lighter peaks can be observed at different sucrose concentrations and probably represent DI particles or aggregations of viral particles.

ml stoppered plastic test - tubes. The cells were then washed gently three times with PBS, saving each wash with the initial inocula. 1M NaOH was then added to each well and left for 5 min to ensure complete solubilization, gentle agitation was sometimes required. The solubilized cells were then placed in plastic test tubes and counted, together with unbound virus on an LKB gamma counter. (Krah and Crowell, 1982; Rubio and Cuesta, 1988; Uncapher et al, 1991 and Kadan et al, 1992)

Inhibition of virus binding was investigated by the pretreatment of the FRhK - 4 cell surface with various enzymes and lectins and the pretreatment of the virus surface with HAV - specific monoclonal antibodies.(see chapters 4 and 5)

2.16 NEUTRALIZATION ASSAY.

The ability of HAV - specific monoclonal antibodies to neutralize the replication of HAV was established in the same way as the titration of HAV by plaque assay except that prior to the addition to the cells, aliquots of virus were incubated with 0.6μg/ml monoclonal antibody (mAb) 813,0.22μg/ml mAb 10.09 and 0.33 μg/ml mAb LSHTM/14H (concentrations of mAbs had yet to be established) for 2 hr at 35°C. Neutralization was considered to have occurred if the number of plaques had been reduced by 50 % or more. (Dimmock, 1984; Hughes et al, 1984)

2.17 HAEMAGGLUTINATION

20μl of PBS [pH 5.5] (30.2mM KH_2PO_4, 119mM NaCl, adjusted to the correct pH with NaOH) was placed into a round - bottom microtitre plate. 20μl of 1%v/v RBC in PBS [pH 5.5] was added to the well, together with an appropriate haemagglutinating dilution of HAV. The plate was then left at room temperature for 1 hr, by which time the RBC would have settled if haemagglutination had not occurred.

The number of haemagglutination units (HAU) was then the inverse of the haemagglutinating dilution.

Inhibition of haemagglutination was investigated by the pretreatment of the RBC cell surface with various lectins and the pretreatment of the virus surface with HAV - specific monoclonal antibodies. (see chapters 3 - 5)(Eckels et al, 1989)

2.18 VIRUS - RECEPTOR COMPLEX FORMATION.

FRhK - 4 cells were incubated in suspension with [^{125}I] - HAV for 2 hr at 4°C in binding buffer. The cells were then washed by centrifugation three times and then solubilized in 1% N - octylglucoside for 15 min on ice. The bound and unbound HAV were separated on a linear 15 - 30% sucrose gradient and fractions containing HAV identified by sandwich ELISA. Alternatively [^{125}I]-HAV was incubated in binding buffer for 2 hr at 4°C with FRhK - 4 cell presolubilized as above. The resulting suspension was then separated on a sucrose gradient as before. These fractions were further investigated by SDS - PAGE. (Mapoles et al, 1985; Krah and Crowell, 1985)

3. HAV BINDING STUDIES

3.1 INTRODUCTION

At the commencement of the project it was generally accepted that the cell tropism of HAV was limited to primate cells (Purcell et al, 1984) but the way in which this tropism is determined was not known. Information regarding binding of HAV to cells was limited to work by Seganti et al., (1987; 1989) which implicated a role for several membrane components including phospholipids and glycolipids in the infection of the cell.

Apart from the observation that HAV must bind to red blood cells in order to agglutinate them (Eckels et al., 1989), the range of cells that the virus was able to bind to was not known. It has been well documented however, that the presence or absence of cell specific membrane molecules determine the cells infected by several viruses. Human immunodeficiency virus (HIV) for instance, infects cells which bear the CD4 molecule (Dalgleish et al., 1984). There was a possibility therefore, that HAV could utilize this mechanism of specific receptor - mediated entry for determining its cellular tropism. If the full binding range of the virus could be determined, then several questions regarding its pathology could then be answered.

Firstly, the route of infection in the host could be completed, since the mechanism by which the virus passes from the gut to the blood and then to the liver had not been confirmed. Poliovirus infects gut cells before causing viraemia and subsequent infection of the upper respiratory tract and CNS, (Bodian, 1955; Sabin, 1956), so there was a strong possibility that HAV may also use gut cells as a primary site of replication.

The ability to identify cell types permitting binding could then be used to support this ' gut replication ' hypothesis. In addition it may also be used to explain a second observation, that patients suffering from fulminant hepatitis A

virus infections who undergo a liver transplant may suffer a reinfection of the transplanted liver, indicating that, *in vivo*, the virus is able to replicate in cells other than hepatocytes (Fagan et al., 1990).

As well as knowing little about of the range of binding tropism HAV exhibited, there was no information regarding other aspects of the cellular receptor for the virus, such as the kinetics of the interaction, and the receptor copy number on permissive and non-permissive cells. This was particularly relevant as it would shed more light upon its distribution (e.g. widespread occurrence of the receptor might imply a common molecule, highly conserved amongst a range of cell types) and may answer questions as to the extremely poor replication of HAV in tissue culture, Wheeler et al., (1986a) observed that HAV is not efficiently uncoated in tissue culture, with a substantial proportion of the inoculum being recovered 12 hours after infection.

A series of binding experiments were therefore devised to investigate these questions.

RESULTS AND DISCUSSION

3.2 BINDING OF HAV TO CULTURED CELL MONOLAYERS

3.2.1 Temperature

The first experiment was designed to optimise the temperature for the binding of virus to cells. This was an important consideration, since the capping of cellular receptors and the resulting reduction in the number of available virus binding sites at 37°C has already been suggested (Zajac et al., 1991).

Therefore any estimation as to the number of receptor sites would be inaccurate if capping of the HAV cellular receptor takes place. In addition, at 37°C membrane proteins are dynamic and are constantly being internalized and recycled. The kinetics of such an experimental arrangement would be highly complex and difficult to interpret. Therefore it would be more convenient if

binding assays could be performed at a temperature at which internalization was reduced to a minimum or abolished altogether.

Therefore a comparison of binding at two temperatures, 4°C and 35°C was performed under the same conditions as described in section 2.15 (Fig. 3.1). The results show that after a two hour incubation more virus is bound at 4°C than at 35°C. There are three possible explanations; Firstly, that receptor capping has indeed taken place at the higher temperature, secondly, that the interaction between the inoculating virus and the cell surface has not reached equilibrium because the virus is being internalized (the iodinated virus proteins would subsequently be recycled and lost from the cell), and lastly that at 35°C the interaction between virus and receptor is not stable with a low resulting binding constant. These three points will be addressed in the discussion.

The conclusion from this experiment was that binding experiments should be performed at 4°C.

3.2.2 Binding Kinetics

Having optimised the binding temperature, the next step was to attempt to saturate an FRhK-4 monolayer with iodinated virus. This was undertaken for several reasons. Firstly it was the most convenient way of ensuring that the binding of HAV to this cell line was specific. If the monolayer proved to be unsaturable this would indicate that the binding observed in these experiments was not necessarily specific , since non-specific binding between a ligand and a cell surface is unsaturable. Secondly the saturation curve obtained from these experiments would confirm whether binding of virus and cell receptor was of a simple 'Michaelis-Menton' type, with one virion binding for every one receptor molecule. This form of interaction would result in data that would fit to an equation based on the Michaelis - Menton equation.

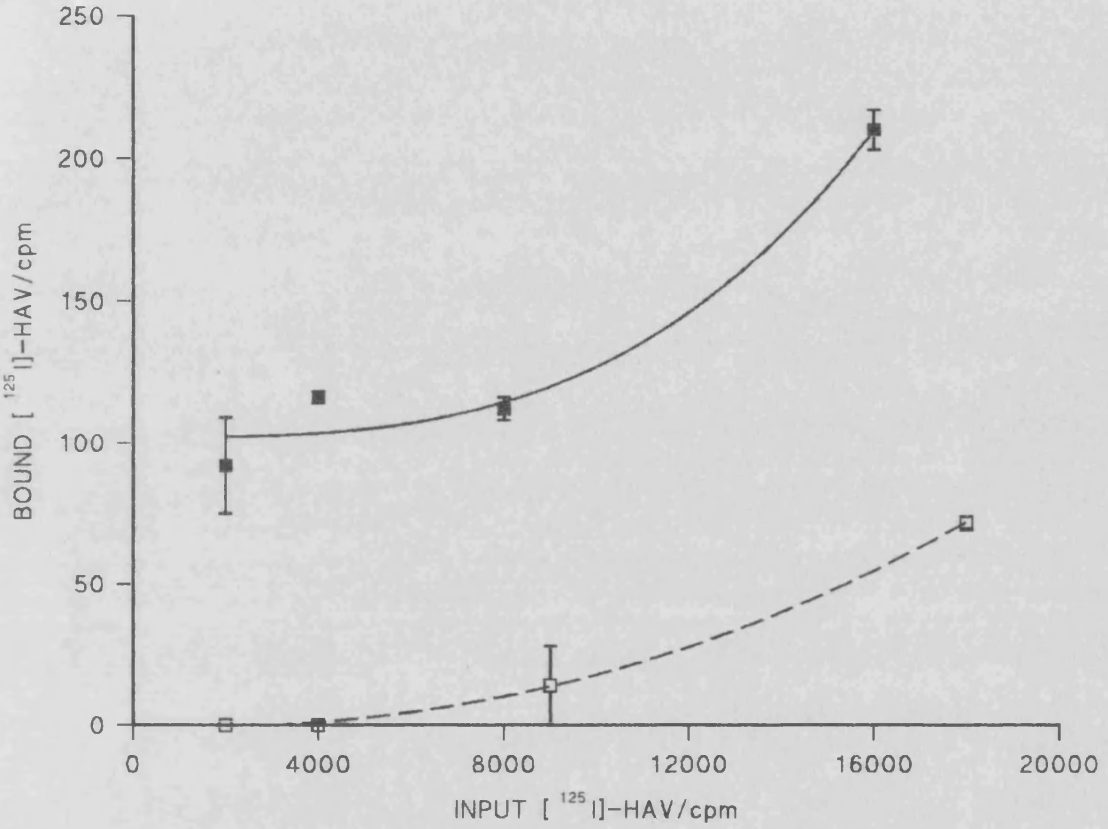


Fig. 3.1 Comparison of ^{125}I - HAV binding to FRhK - 4 cells (2.5 x 10^5 cells /datapoint) at 4°C (—ν—) and 35°C (--□--) for 2 hrs, in binding buffer [1.8 mM Ca^{++}]. Bars represent range of duplicate results obtained. Curves described by equation (3) in section 3.2.2 are fitted to the data. Specific activity of the virus is 2.2 x 10^6 cpm/μg.

$$B = \frac{B_{max} \cdot I}{I + k_{0.5}} \qquad (1)$$

Where, B = bound [^{125}I]-HAV, I = input [^{125}I]-HAV and $K_{0.5}$ = input virus at 0.5 x B_{max}

Lastly, this type of binding assay would give an indication of the number of binding sites on each permissive cell, which may in turn indicate how common a molecule the cellular receptor is. For instance, the number of sialic acid molecules on a mammalian cell surface will be very high, whereas CD4 molecule numbers may be very low.

Fig. 3.2 shows a plot of the data obtained from the saturation of an FRhK-4 cell monolayer. Having attempted to fit a curve described by the Michaelis - Menten equation, it was obvious that the interaction under investigation did not follow classical Michaelis-Menten kinetics.

Instead of a rectangular hyperbola, the data in Fig. 3.2 seems to follow a sigmoidal curve.

This type of curve can be indicative of an allosteric mechanism, whereby the binding of one ligand to a receptor molecule increases the affinity of the same receptor for a second ligand. We therefore attempted to fit a curve to the data, which describes such an interaction using the Pfit curve fitting program supplied with FigP (Biosoft) scientific graph package (Fig. 3.2) The curve is described by the following equation,

$$B = \frac{B_{max} \cdot I^h}{I^h + k_{0.5}} \qquad (2)$$

Where, B = bound [^{125}I] - HAV, I = input [^{125}I] - HAV, $k_{0.5}$ = input [^{125}I] - HAV at 0.5 x B_{max} and h = Hill coefficient.

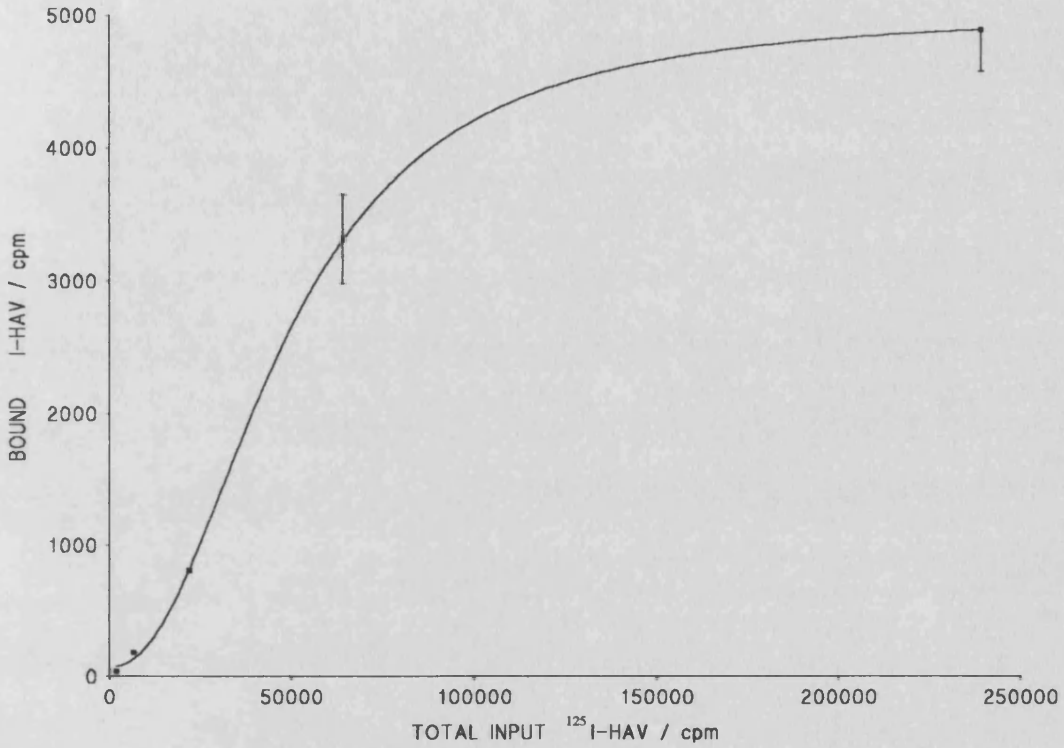


Fig. 3.2 Saturation of an FRhK-4 cell monolayer (2.5 x 10^5 cells/datapoint) with [^{125}I] - HAVat 4°C for 2hr in binding buffer [1.8mM Ca^{++}]. A curve described by the Hill equation for allosteric interactions is fitted to the data. Bars represent range of duplicate results.

B_{max} = 5039 cpm

$k_{0.5}$ = 47716 cpm

h = 2.17

As can be seen the curve described by this equation fits the data well, though no definitive 'closeness of fit' can be given.

To confirm that HAV binding to FRhK-4 cells did not follow classical Michaelis-Menten binding kinetics, a second plot was constructed (Fig. 3.3). This Scatchard plot (Scatchard, 1949) will linearize single-site binding kinetics data by plotting the following,

$$\frac{\text{bound } [^{125}\text{I}] \text{ - HAV}}{\text{free } [^{125}\text{I}] \text{ - HAV}} \text{ AGAINST bound } [^{125}\text{I}] \text{ - HAV} \qquad (3)$$

As can be seen in fig. 3.3, however, this plot is not linear and thus confirms that the interaction of HAV with its cellular receptor does not follow simple kinetics.

Initially, the mechanisms behind such kinetics were difficult to envisage since the interaction of a ligand of $\approx 8 \times 10^6$ Da with a cell surface receptor was under study. Two possible explanations have been considered. Firstly, if the aggregation of HAV is in fact a dynamic event, with dissociations as well as associations, and the affinity of virion for virion is greater than that of receptor for virion, then it is possible that the binding of a single virion to the cell surface apparently increases the affinity of a second virion for the cell surface, via the bound virion.

The second explanation is based on data recently obtained by Anderson et al (Meeting Abstract, 1992). This group have isolated a putative cell binding site for HAV, showing it to be a homodimer of 200kDa. They have also shown that HAV is able to bind to the monomer as well as the dimer. There is now scope

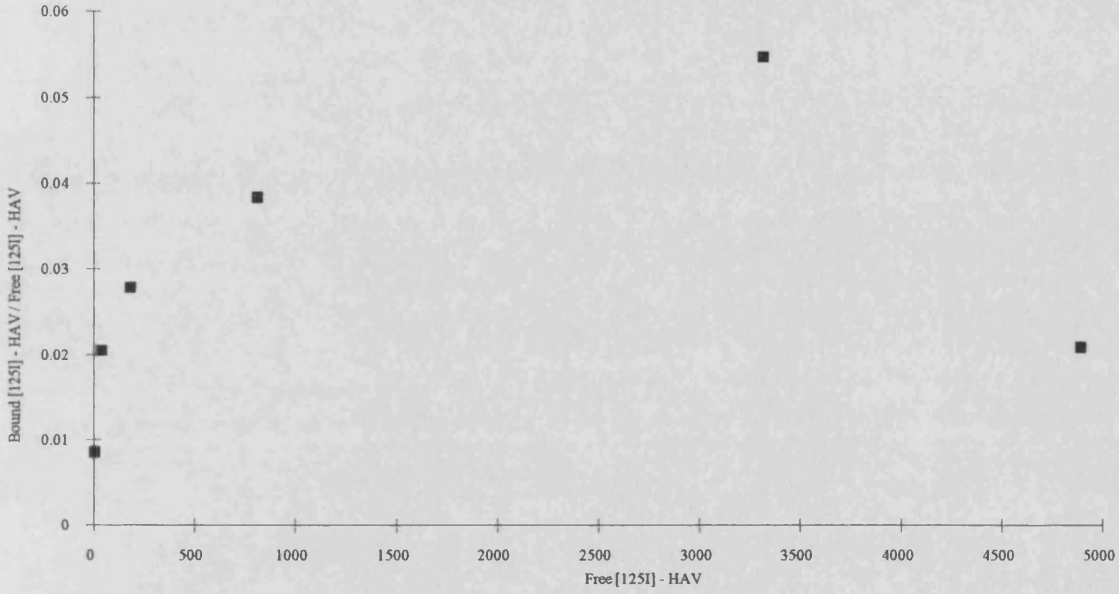


Fig. 3.3 Scatchard plot of the saturation of an FRhK-4 cell monolayer ($2.5x10^5$ cells/datapoint) with [^{125}I]-HAV at 4°C for 2hr in binding buffer [1.8 mM Ca^{2+}]. Specific activity is 2.2×10^6 cpm/μg.

to explain an allosteric interaction of HAV and its receptor, since if their findings are correct there are two binding sites on each receptor dimer.

3.2.3 Binding Tropism of HAV

Having confirmed that a specific interaction is taking place, the next phase of the project was to examine the range of the binding tropism of the virus. To this end, under exactly the same conditions, the binding of [^{125}I] -HAV to three different cell lines, FRhK-4, CHO and WRC was compared, as a prelude to more extensive studies. As can be seen in figs.3.4 - 3.5, the binding curves for the CHO and WRC cell lines show equal or greater binding to that of FRhK-4 cells. This has several implications. Firstly, if the possession of a particular binding molecule was the sole determinant of tissue tropism, it would be expected that binding would only occur to FRhK-4 cells and not to CHO and WRC. This is plainly not the case so other determinants of tropisms must be considered. Tissue tropism may still be determined at the cell surface, but not solely by the presence or absence of certain binding molecules. Herpes simplex virus, as an example, will bind to cells bearing the heparan sulphate moiety, which is common to all mammalian cell types. Tissue tropism however, is thought to be governed by the possession of a second type of plasma membrane molecule, which is involved in internalization of the virus (Johnson et al., 1990). The second possibility is that HAV is internalized by all cells which bear its cellular receptor, and that it is the intracellular elements of its replication which determine whether progeny viruses will be produced. Examples of such determinants include endosomal pH or host cell ribosome specificity. The investigation of the range of binding tropism of HAV was extended to a haemagglutination assay (Fig. 3.6) where HAV was found to bind to red blood cells. The notable feature of this is that the haemagglutination assays were performed in PBS at a pH of 5.5, devoid of divalent cations, which have been shown to be essential for the binding of HAV to permissive cell lines at a pH of 7.4 (Zajac et al., 1991).

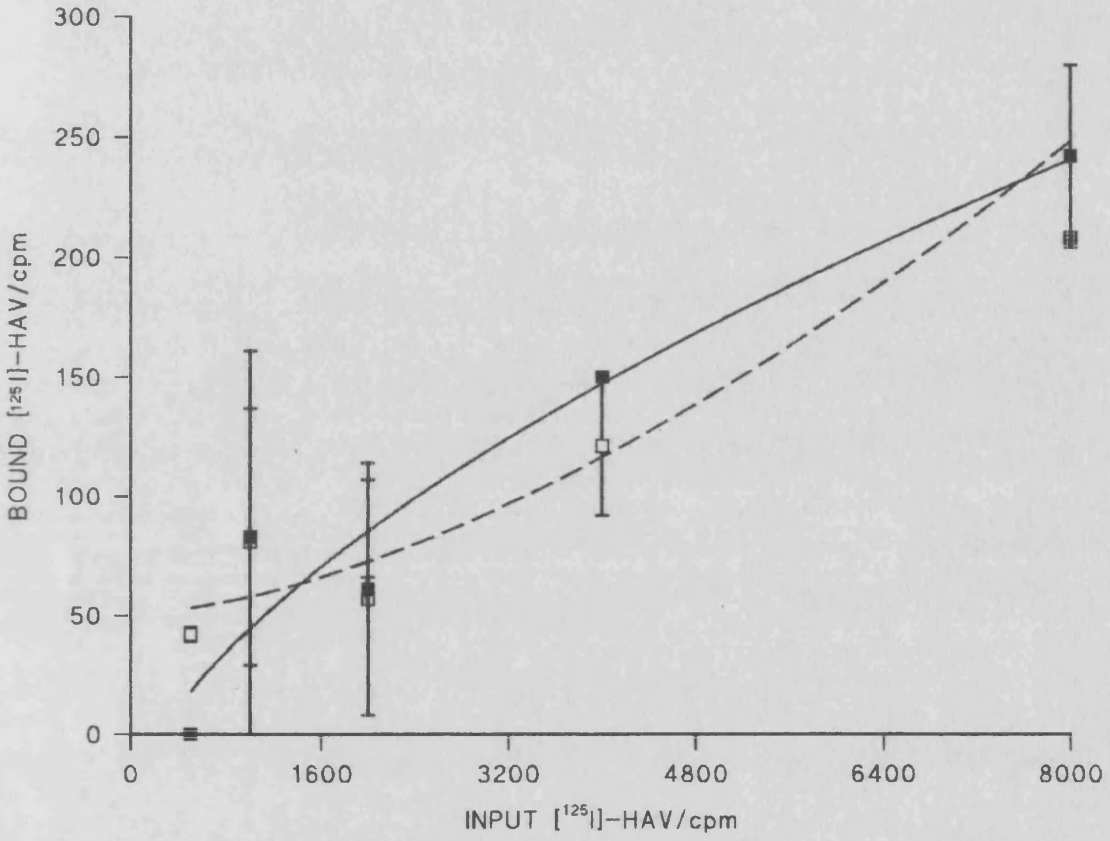


Fig. 3.4 Comparison of the binding of [^{125}I]-HAV to FRhK-4 (—v—) and CHO (--□--) cells (2.5 x 10^5 cells / datapoint) at 4°C for 2 hr in binding buffer [1.8 mM Ca^{2+}]. Bars represent range of duplicate results. Curves descibed by equation (3) given in section 3.2.2 are fitted to the data.

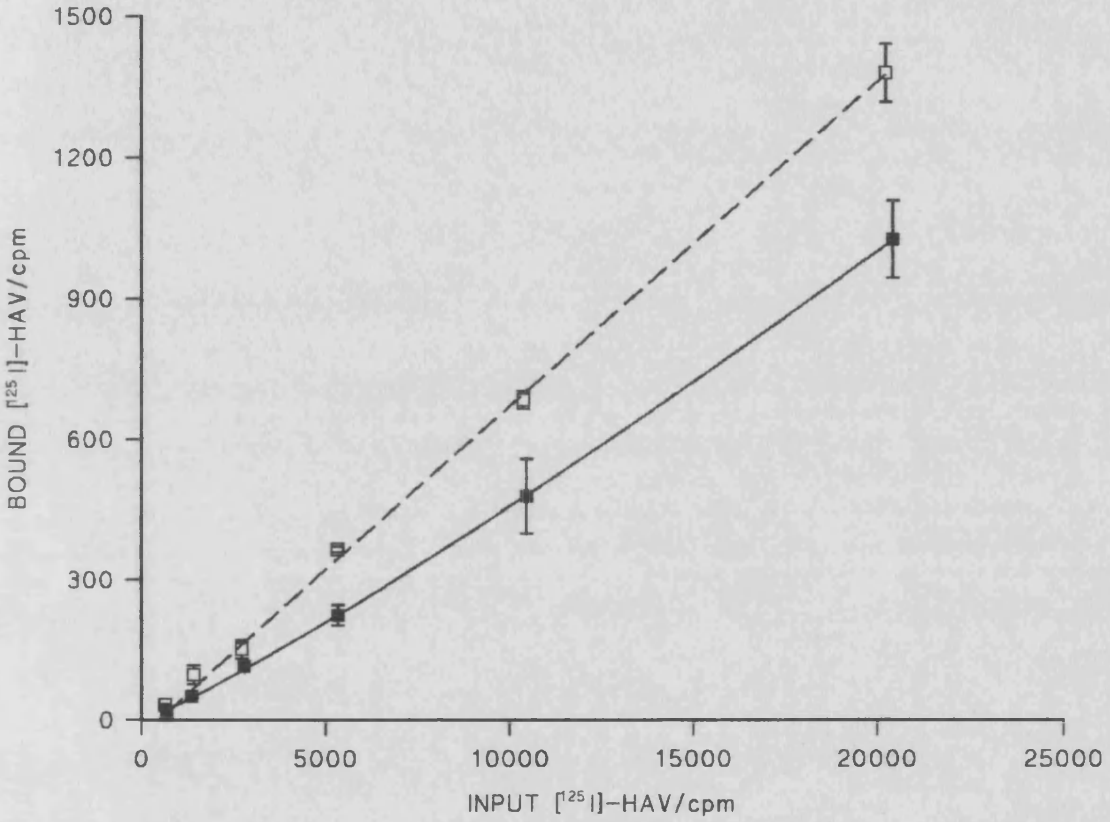


Fig. 3.5 Comparison of the binding of ^{125}I-HAV to FRhK-4 (—v—) and WRC (--□--) cells (2.5 x 10^5 cells/datapoint) at 4°C for 2hr in binding buffer [1.8 mM Ca^{2+}]. Bars represent range of duplicate results. Curves described by equation (3) in section 3.2.2 are fitted to the data.

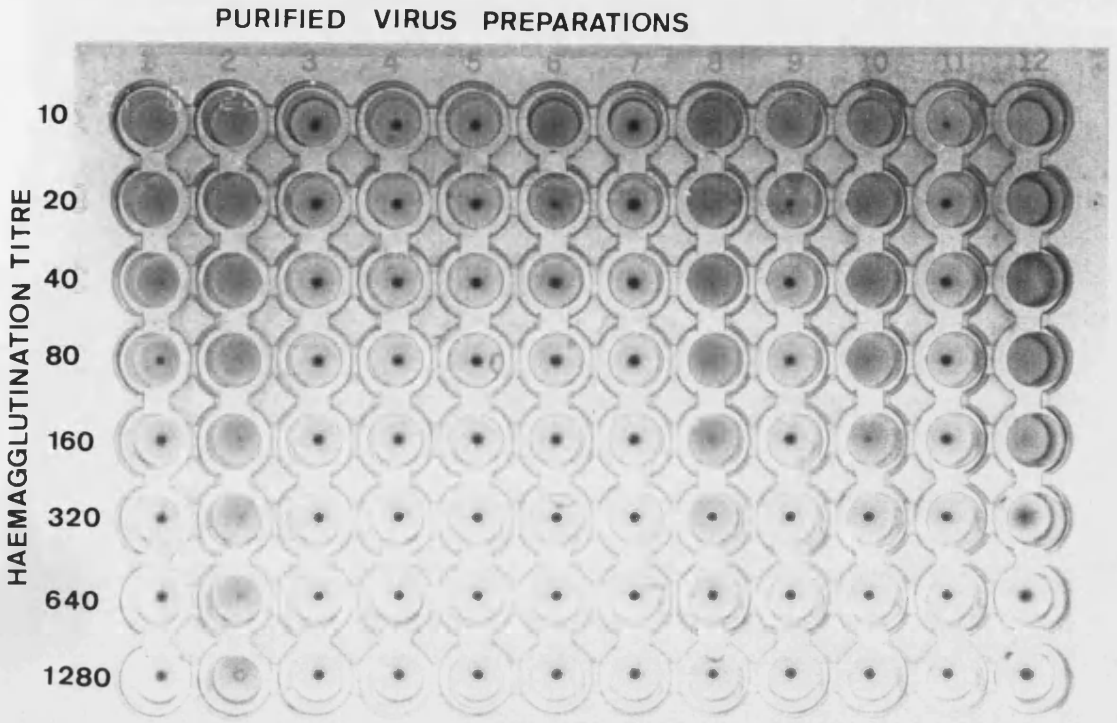


Fig. 3.6 Agglutination of red blood cells with various preparations of purified HAV in PBS [pH 5.5] at room temperature.

The implications of all of these points will be considered in the discussion (chapter 6).

3.3 VIRUS - RECEPTOR COMPLEX

As well as gaining some knowledge about the interaction of HAV with various cell lines, attempts were made to purify the receptor. This would allow study of the structure of the receptor such as N-terminal sequence determination, which are not possible with cell bound proteins. At the start of the project there was very little information available about the makeup of the receptor in terms of molecular weight, pI and other parameters which would make purification possible. The only two techniques available were therefore the production of a monoclonal antibody to the receptor using a cell protection assay as a screen or to use the virus itself as an affinity ligand to co-purify the receptor with the virus (Mapoles et al., 1985). Production of a monoclonal antibody was considered to be too time consuming, since the only available source of receptor was whole cells. This would mean screening numerous clones in order to obtain one directed against the epitopes on the receptor. As a result, the latter technique was used.

Since the amount of purified virus available was limited, it was unacceptable to use any method which did not make the best use of the virus. Mapoles et al., 1985 perfected a method for purifying the cellular receptor of Coxsackie B3 virus (another Picornavirus), which entailed the separation, on a sucrose gradient, of free virus from virus bound to its cellular receptor. It is this procedure upon which the attempted copurification of the cellular receptor for HAV was based.

[^{125}I] - HAV was incubated in binding buffer [1.8mM Ca^{2+}] for 2 hr at 4°C, with FRhK - 4 cells which had been solubilized in 1% n-octylglucoside and left on ice for 15 min. The cell/virus suspension was then carefully layered onto a continuous 15 - 45% sucrose gradient, and ultracentrifuged for 3 hr at 4°C at

86,500 x g using a Beckman SW27 ultracentrifuge rotor. Fractions of 0.5ml were collected from the gradient, and the presence of [^{125}I] - HAV was detected by counting on an LKB gamma counter. Figs. 3.7 - 3.9 show the resulting gradients, and indicates that a proportion of [^{125}I]-HAV has sedimented at a faster rate than control virus alone. There can be no doubt that there is viral antigen present in this higher molecular weight fraction, since the viral proteins have been radiolabelled with [^{125}I] which suggests that the virus has associated with some component of the FRhK-4 cell membrane. Points A and B represent two possible positions where a complex of virus and receptor may have occurred. Anderson et al, 1992 (Meeting abstract) proposed that a virus - receptor complex under these conditions would appear as a shoulder on the main uncomplexed virus peak (peak B). Point A may represent an aggregation of HAV associated with, and mediated by cellular components. Though these methods can certainly be improved by optimising conditions for the production of virus-receptor complex and subsequent isolation, the results indicate that this is still a valid route by which the receptor may be isolated.

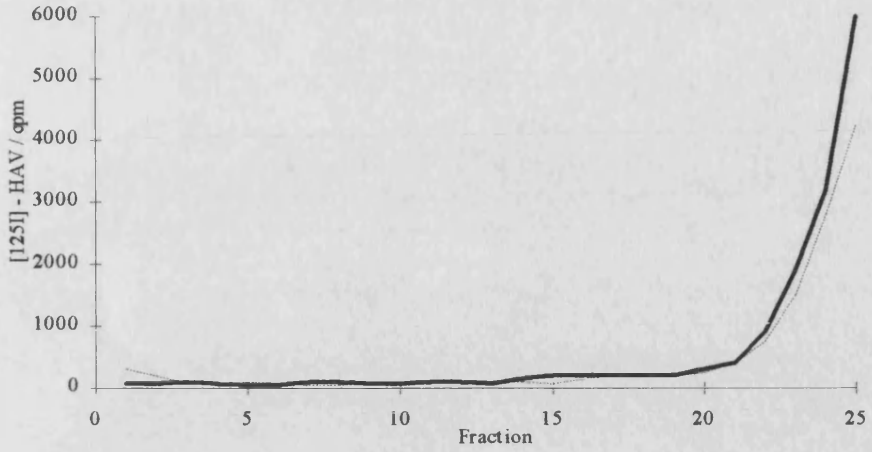


Fig. 3.7 Profile of a sucrose gradient to separate ^{125}I-HAV and ^{125}I-HAV-receptor complex having incubated labelled virus with BSC-1 cells (solubilized in 1% N-octylglucoside) in binding buffer for 2 hr at 35°C. (·····) represents virus receptor complex isolation, (—) represents virus only control.

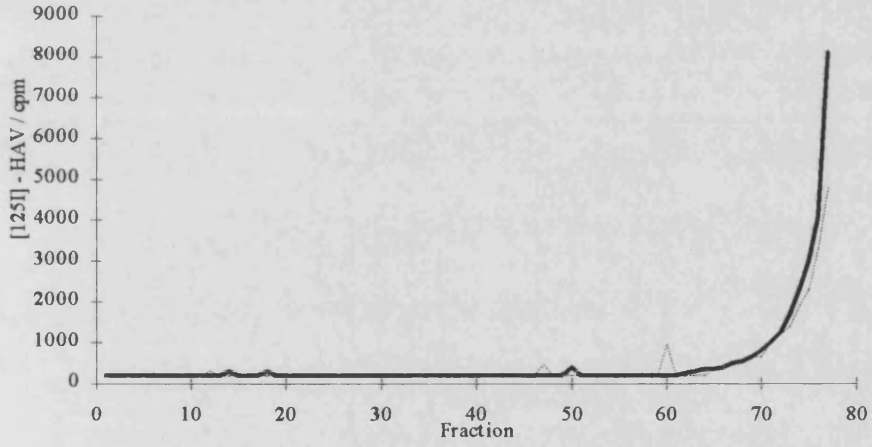


Fig 3.8 Profile of a sucrose gradient to separate ^{125}I-HAV and ^{125}I-HAV-receptor complex having incubated labelled virus with BSC-1 cells (solubilized in 1% N-octylglucoside) in binding buffer for 2 hr at 4°C. (·····) represents virus receptor complex isolation, (—) represents virus only control.

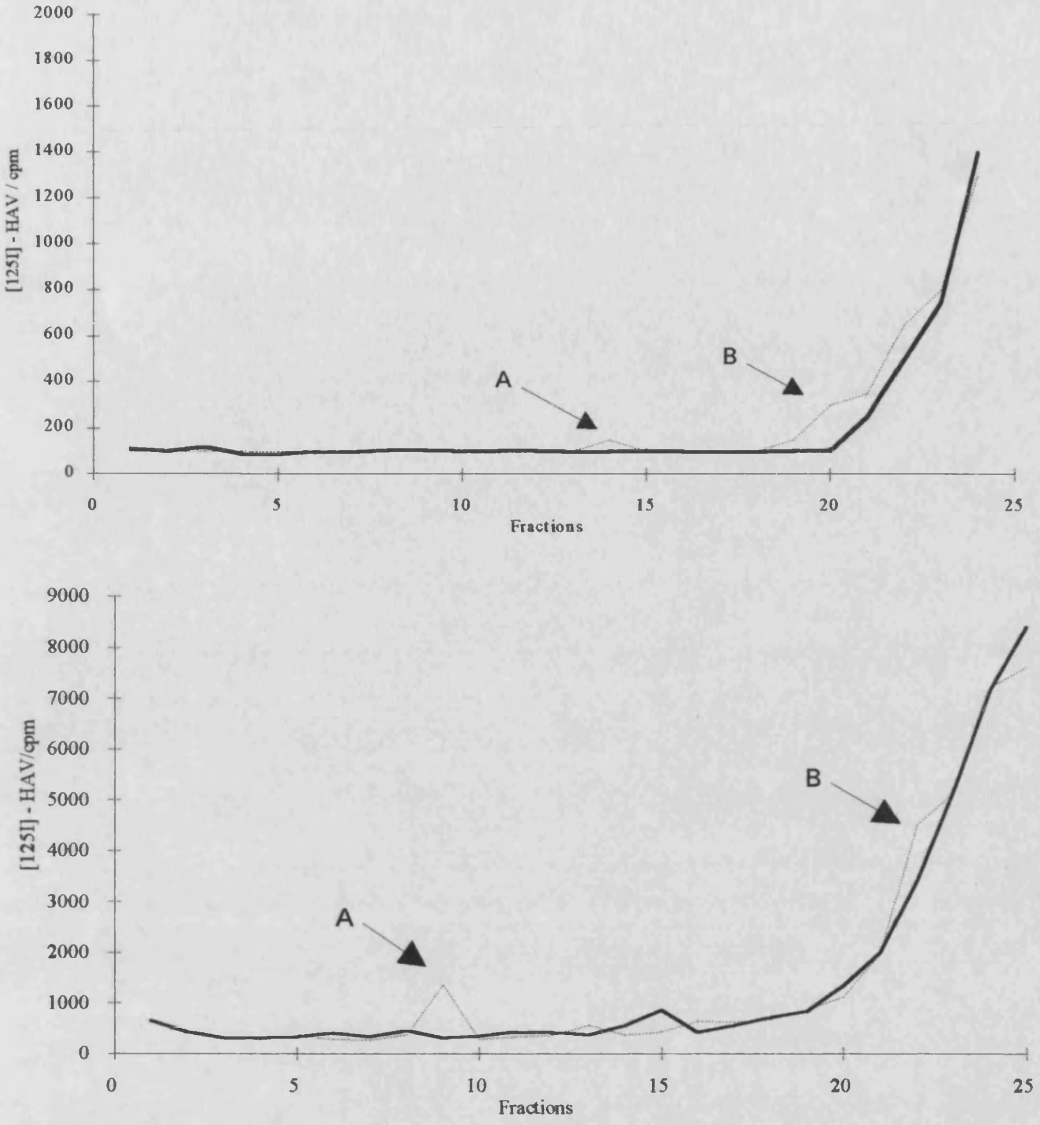


Fig. 3.9 Profiles of a sucrose gradient to separate ^{125}I-HAV and ^{125}I-HAV-receptor complex having incubated labelled virus with FRhK-4 cells (solubilized in 1% N-octylglucoside) in binding buffer for 2 hr at 4°C. (·····) represents virus receptor complex isolation, (—) represents virus only control. Points A and B represent possible fractions where a virus-receptor complex may reside.

4. HAV - SPECIFIC MONOCLONAL ANTIBODIES

4.1 INTRODUCTION

Previous work on this area of HAV virology is far more extensive than has been described for the kinetics of HAV binding or for the structure of the cellular receptor. This was mainly due to the fact that a effective vaccine for HAV had not been established, so it was important to gather information regarding the interaction of HAV with HAV - specific monoclonal and polyclonal sera. There were many HAV - specific antibodies available in various different laboratories (Ping and Lemon, 1992), though only three monoclonals have been investigated in this study; LSHTM /14H, 813 and 10.09 (Crevat et al, 1990), and a convalescent serum IgG fraction. These monoclonal antibodies had been previously used by Ping and Lemon, (1992) to establish the antigenic structure of the virus, by the production and sequencing of mutants which had escaped the antibodies' neutralization properties. In the process they had gathered data regarding the cross reactivity and neutralization potential of the antibodies. They had shown that 813 and 10.09 reduced the binding of an iodinated polyclonal antibody to about 52%, whereas 14H reduced its binding to 64%. They also showed that 813 and 10.09 almost completely neutralized HAV in a radioimmunofocus assay, whereas 14H could only reduce radioimmunofoci by up to 50%.

Another important observation regarding the antigenicity of HAV is that there only appears to be one wild-type serotype virus (Ping and Lemon, 1992), indicating that to escape antibody neutralization by polyclonal sera, the virus would have to mutate in such a way that it was no longer viable. This in turn suggests that the site of neutralization for HAV is extremely important for replication.

There was however, no detailed information regarding the mechanisms behind the neutralization of HAV, although the subject of virus neutralization had been addressed by many different groups (for review see Dimmock, 1984). One possible mechanism that had been put forward was virus neutralization by antibody - mediated inhibition of binding. This would occur by the antibody competing with the cellular receptor for binding to the virus, and could be used to explain why HAV can only maintain one serotype virus.
It was decided therefore that a series of experiments would be undertaken to investigate whether this was true for HAV.

RESULTS AND DISCUSSION

4.2 NEUTRALIZATION OF HAV

Firstly, the ability or inability of the three monoclonal antibodies (MAbs) LSHTM/14H, 813 and 10.09 to neutralize the virus was confirmed by incubating infectious virus with 1.5 μg/ml of each of the antibodies in PBS for 2hr at 35°C, prior to the addition of the virus to a standard plaque assay. Table 4.1 shows the reduction in plaque numbers after the virus had been preincubated with MAbs 813 and 10.90. In contrast, no such reduction is observed in the virus preincubated with MAb LSHTM/14H; indicating that this monoclonal antibody is unable to neutralize HAV. This agrees with the results obtained by Ping and Lemon, (1992), and indicates that these antibodies represent a system by which the site of virus neutralization may be ascertained.

4.3 COMPETITION RADIOIMMUNOASSAY.

If the virus is neutralized by the binding of antibodies to the cell binding site, it would be reasonable to assume that the non-neutralizing antibody, 14H fails to neutralize because it does not interact with these sites. There would therefore

Monoclonal Antibody	Plaque Number
Control	27 (± 8)
14H	22 (± 2)
813	1 (± 1)
10.09	2 (± 1)
Mock Infected	0

Table 4.1 Reduction of plaque number by the preincubation of infectious HAV with neutralizing and non-neutralizing monoclonal antibodies prior to inoculation of an FRhK-4 cell monolayer.

be a possibility that, whereas 813 and 10.09 will compete for binding to the virus capsid, 14H will not compete with either of the other two antibodies. This would actually be a simplistic view of the possible events since a non-neutralizing and neutralizing antibody may still compete with each other whilst not necessarily both blocking binding of virus to cell.

To ascertain whether 14H, 813 and 10.09 would compete for binding to the cell, a competition binding assay was performed (see 2.10) between [^{125}I]-10.09 and each of the monoclonal antibodies and an anti-HAV convalescent IgG fraction. Fig 4.2 demonstrates how MAb 813 and 10.09 reduced binding of [^{125}I]-10.09 to around 48%, whereas LSHTM/14H reduced the binding of the labelled antibody to only 76%. The convalescent IgG fraction also reduced binding by around 50%. Since the concentrations of all the monoclonal antibodies in the assay are the same, these results suggest that monoclonal antibodies 813 and 10.09 compete for the same epitope on the virus capsid. The result for monoclonal antibody LSHTM/14H, however, suggests that it is binding to an epitope sufficiently distant from the binding sites of 813 and 10.09 to allow the labelled antibody [^{125}I]-10.09 to still bind. These results agree, in principle, with those of Ping and Lemon, (1992) which indicated that 14H did not interfere with the binding of a polyclonal antibody to the same extent as 813 and 10.09 (14H reduced binding to 64% whereas 813 and 10.09 reduced binding to 52%). Both the data of Ping and Lemon, 1992 and the data presented here therefore agree with the general hypothesis of discrete, (but overlapping?) epitopes utilized by neutralizing and non-neutralizing antibodies. It must be emphasized however, that Ping and Lemon, 1992 attempted to block the binding of a polyclonal antibody with 813, 10.09 and 14H, as in contrast to these results, which describe the inhibition of binding of the neutralizing monoclonal antibody 10.09. The primary consequence of this is that residual binding of the iodinated polyclonal would still be expected even after saturation of the virus with monoclonal antibodies 813 or 10.09.

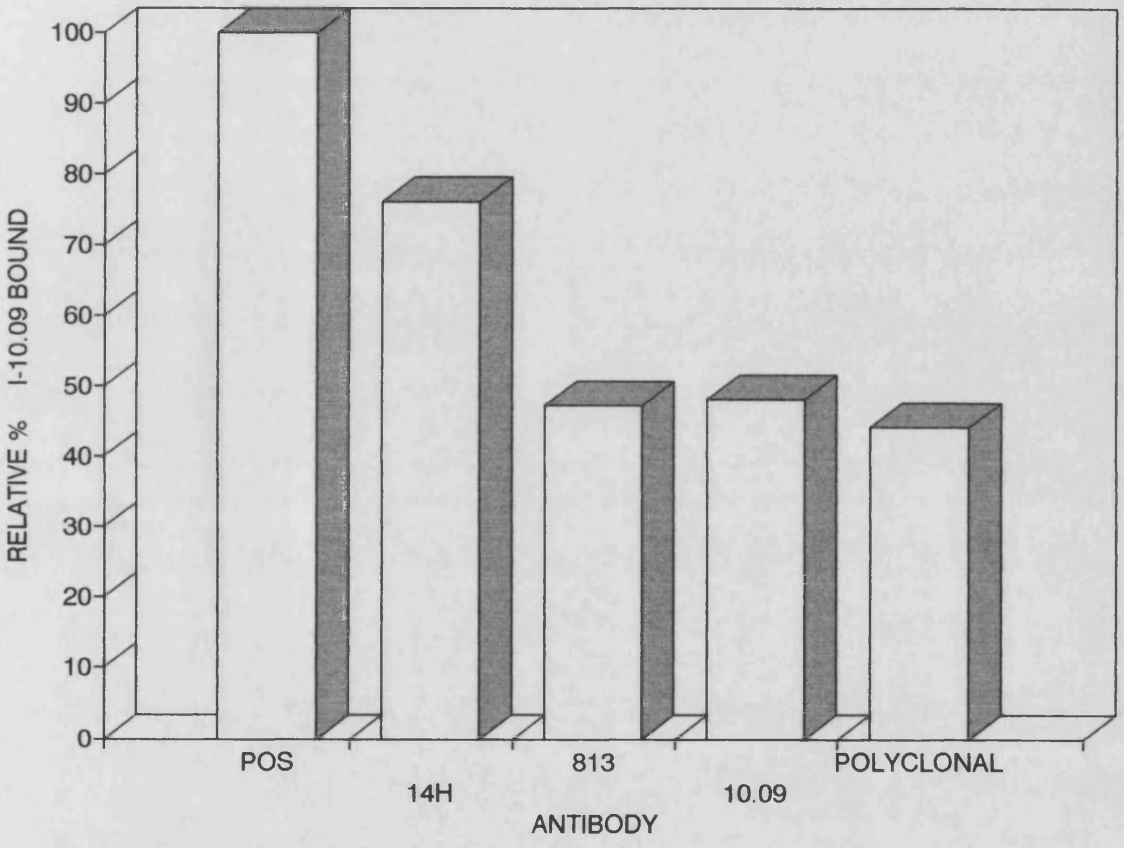


Fig. 4.2 Competition radioimmunoassay between ^{125}I-labelled HAV - specific monoclonal antibody 10.09 and HAV - specific antibodies 14H, 813, 10.09 and an IgG fraction of convalescent sera to HAV. Maximal binding (100%) 1000cpm.

Having obtained these results it was important to confirm the hypothesis of antibody neutralization at the cell binding site, by directly measuring the effect of these antibodies on HAV - cell interactions.

4.4 INHIBITION OF HAEMAGGLUTINATION

Having established the neutralization properties of the three HAV - specific monoclonal antibodies, and how they apparently behaved in a competition assay, the next step was to investigate the ability of the monoclonal antibodies to interfere with virus binding to cells. As has been reported previously, HAV binds to various different cell lines. In the case of red blood cells this causes haemagglutination. This represents a possible model for the investigation of virus binding to cells, and therefore can be used to investigate the inhibition of binding by monoclonal antibodies.

2 haemagglutination units (HAU) of HAV were pre - incubated with doubling dilutions of MAbs LSHTM/14H, 813, 10.09 or a convalescent sera IgG fraction, for 2hr at 37°C before being added to RBC in a standard haemagglutination assay (see 2.18).

Fig 4.3 and table 4.4 reveal how MAbs 813 and 10.09 have inhibited haemagglutination to a titre of 480 and 1920 respectively by inhibiting binding of the virus to the red blood cells. MAb LSHTM/14H however, did not inhibit haemagglutination even at a titre of less than 15 indicating that it is unable to interfere with virus binding to red blood cells.

The suggestion that neutralizing and non - neutralizing antibodies have different effects on HAV - mediated haemagglutination has not previously been observed and adds further evidence towards the hypothesis that neutralizing antibodies neutralize by binding to an epitope on the virus sufficiently close to, or on, the cellular binding site. This is reinforced by the observation that the non - neutralizing antibody has no effect on binding of virus to RBC, and so is probably distant from the cellular binding site.

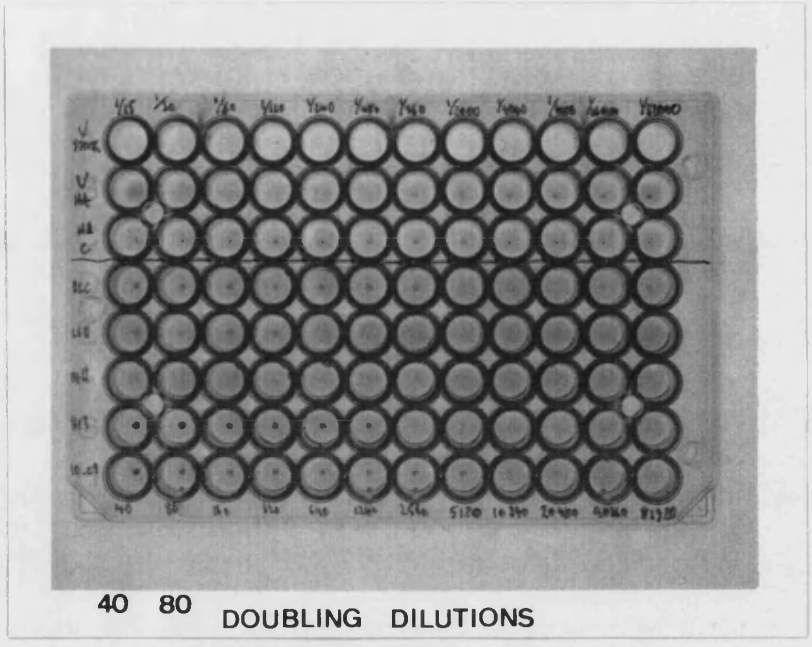


Fig. 4.3 Inhibtion of HAV - mediated haemagglutination by preincubation of HAV samples with HAV - specific antibodies.

HAV - specific antibody	Antibody concentration (mg/ml)	Haemagglutination Inhibition titre
Monoclonal 14H	0.33	<15
Monoclonal 813	0.60	480
Monoclonal 10.09	0.22	1920
Convalesent IgG fraction	0.88	960

Table 4.4 Inhibtion of HAV - mediated haemagglutination by preincubation of HAV samples with HAV - specific antibodies.

4.5 INHIBITION OF BINDING TO A PERMISSIVE CELL LINE.

The results obtained in section 4.4 suggested that the neutralization of HAV by monoclonal antibodies takes place as a result of those antibodies sharing their epitopes with the cell binding site of the virus. Though the use of red blood cells represents a convenient way to investigate virus - cell interaction, it is still necessary, ultimately to confirm the results by attempting to inhibit virus binding to a cell line known to be infected by HAV in tissue culture.

To achieve this, [^{125}I]-HAV was incubated with 1.5μg/ml of each of the three monoclonal antibodies, for 2 hr at 37°C. Following this a standard binding assay was performed (see section 2.15).

As can be seen in Fig. 4.5, each of the monoclonal antibodies investigated has all but abolished binding of HAV to the FRhK-4 cells.

One possible explanation for the apparent contradiction between the haemagglutination inhibition assays and these binding inhibition assays is that the receptor present on red blood cells is not the same as on FRhK-4 cells; as is the case with influenza virus, where haemagglutination inhibition by monoclonal antibodies occurs but inhibition of binding to permissive cells does not. Though, with the available data it is difficult to construct a model for these events, it is conceivable that the receptor on FRhK-4 cell is less sterically available than the receptor on red blood and other cell types. As a result, though the presence of monoclonal antibody 14H interferes with virus binding to FRhK-4 cells, perhaps the greater availability of the red blood cell receptor means that the hindering action of this monoclonal antibody is not so evident. The action of monoclonal antibodies 813 and 10.09 is as would be predicted, apparently interfering with binding by attaching to the cell binding site of the virus or interfering with the function of this site by allosteric effects.

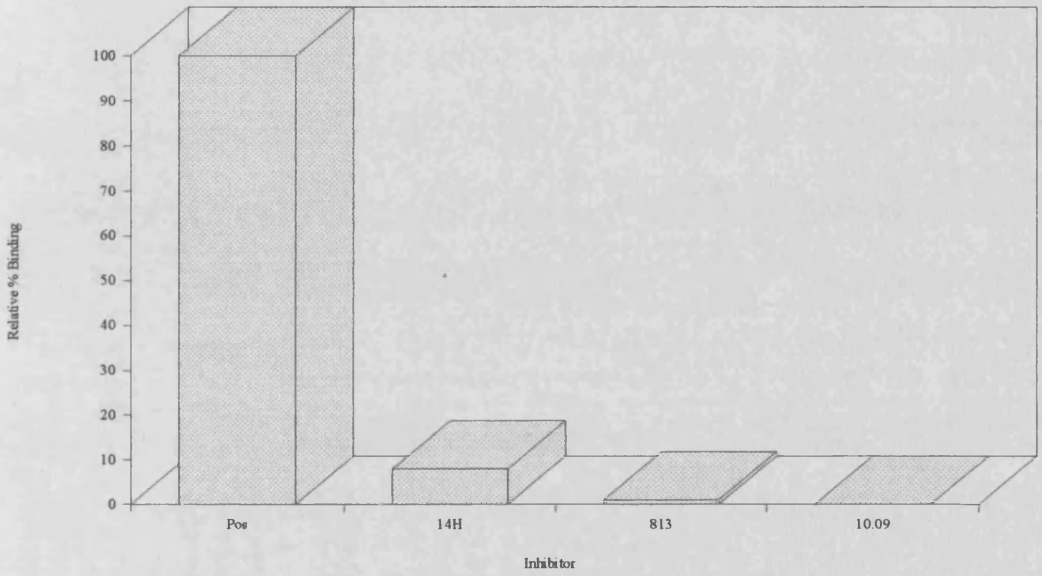


Fig. 4.5 Inhibition of ^{125}I-HAV binding to an FRhK-4 cell monolayer by the preincubation of the virus with HAV - specific monoclonal antibodies 14H, 813 and 10.09. Maximal binding of positive control (100%) 85cpm.

5. BINDING INHIBITION STUDIES

5.1 INTRODUCTION

One way of elucidating the structure of a virus's cellular receptor is to treat the receptor or the virus in a way which interferes with the binding. This may mean treating the whole cell or cell membrane with enzymes, or incubating them with compounds which will compete with the virus for the binding site. Alternatively it may involve incubating the virus with compounds which compete with the cellular receptor for binding of the virus, which may also give clues as to the structure of the receptor.

This approach had been successfully used for several virus receptors. The receptor for influenza virus, sialic acid, was discovered by treating red blood cells with neuraminidase prior to a haemagglutination assay, resulting in complete inhibition of haemagglutination (Burness and Pardoe, 1981).

The primary binding site for herpes simplex virus, heparan sulphate, was determined when it was observed that heparin bound to the virus and inhibited binding and infection (WuDunn and Spear, 1989).

As was mentioned in chapter 3, Seganti et al., (1987; 1989) used these methods to gather a limited amount of information as to the structure of the cellular receptor for HAV. This group found that the virus binding site on Frp/3 cell membranes was sensitive to trypsin, phospholipase and β - galactosidase (Seganti et al., 1987), whilst binding could also be inhibited by incubating the virus with various membrane lipid components, specifically those bearing galactose residues (Seganti et al.,. 1989). The conclusion at the time was that the receptor for this virus is extremely complex, with proteins, phosphatidlyserine, phosphatidylethanolamine and galactose participating in virus binding. Unfortunately these studies only detected inhibition of binding by measuring inhibition of infection (fluorescence - linked immunoassay). This

did not, however, address the question of whether the initial virus binding site is the only component required for virus infection of permissive cells. In these studies, this question was met by investigating inhibition of virus binding rather than inhibition of virus infection. This involved treatment of the cell surface with various enzymes, and incubation of virus with compounds which mimic the virus binding sites on the cell surface. In addition, the cell surface was incubated with lectins. Lectins are naturally occurring proteins which bind to specific short oligosaccharide sequences, and can therefore be used to determine what, if any, sugars take part in virus binding. Lectins were used by Krah and Crowell (1985) to inhibit the binding of group B Coxsackieviruses to solubilized HeLa cell membranes.

5.2 ENZYMATIC INHIBITION OF BINDING

Three enzymes were used to treat the surface of the cell and subsequently observe the effect on binding. Trypsin was the first choice, to confirm the involvement of proteins, since trypsin, a serine protease, specifically cleaves proteins at arginine and lysine residues. The second, neuraminidase, was used to cleave off sialic acid residues on glycoproteins, to determine whether these moieties were involved, a common occurrence with other viruses such as influenza virus. Finally endoglycosidase F was used, since this enzyme specifically removes oligosaccharide residues from N - linked glycoproteins and so would indicate a role for glycoproteins in the interaction.
10^6 FRhK-4 cells were scraped into cold PBS, spun at 1000 rpm for 2 min, and washed twice to remove residual FCS. The cells were then resuspended in the following enzyme solutions; Vibrio cholerae neuraminidase (0.1U/ml) (Boehringer Mannheim, Mannheim, Germany), for 2.5 hr at 37°C in PBS; Endoglycosidase F (0.4 U/ml) (Boehringer Mannheim), for 2.5 hr at 37°C in PBS; or trypsin (0.5 mg/ml) for 10 min at room temperature. Following three washes, the cells were incubated with 8000 cpm of [^{125}I] - HAV for 2 hr at

4°C in binding buffer . The virus was then removed, but retained, as were the subsequent three washes. The amounts of bound and unbound virus were then established by counting in an LKB gamma counter. Table. 5.1 shows that the digestion of proteins by trypsin inhibits HAV binding which suggests that proteins play a part in the binding of HAV to its target cell (in this case FRhK-4 cells). It does not however, provide any details as to the type of protein it may be. The fact that binding of virus is not completely abolished is almost certainly due to the incomplete removal of proteins from the cell surface. This may have occurred for several reasons. Firstly, since the complete removal of proteins would have resulted in the lysing of the cell, which in turn would have flawed the experiment, a lesser degree of deproteination was considered acceptable. Secondly, because of the autodegradative properties of trypsin, it may not have removed all proteins from the cell surface in the short incubation period. The third, and most contentious possibility, is that HAV relies, for binding, on moieties that are present on proteins, but are also present on other cell surface molecules, such as certain sugar residues. The neuraminidase result proved far from conclusive but suggests that sialic acid does not play a part in virus binding. The result of endoglycosidase F pretreatment of the cell surface suggests that the receptor protein is not an N - linked glycoprotein. This result however, may be misleading as this enzyme may, in some cases, work poorly with folded proteins.

5.3 INHIBTION OF HAEMAGGLUTINATION (HA)

Following the results of the enzyme pretreatment of the cell surface, more detailed analysis of the relevant residues on the cell surface was undertaken, with the use of lectins. Lectins specifically recognize and bind to sugar residues, and together with the ability of HAV to bind to and agglutinate red blood cells, could be used to determine more information about the role of

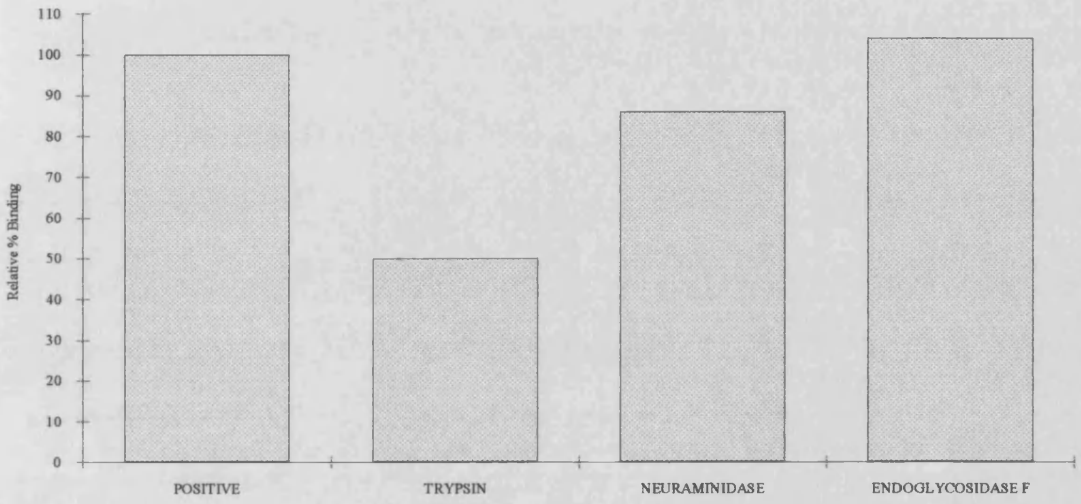


Fig. 5.1 Inhibition of ^{125}I-HAV binding to FRhk-4 cells by enzyme pretreatment of the cell surface. FRhK-4 cells were treated with 0.5mg/ml trypsin for 10 min at room temperature, Vibrio cholerae neuraminidase at 35°C for 2.5 hr or endoglycosidase F at 35°C for 2.5 hr and then incubated with 8000 cpm ^{125}I-HAV at 4°C for 2 hr in binding buffer.

sugar residues in binding. This was accomplished in a series of haemagglutination inhibition (HAI) assays.

Prior to haemagglutination, the red blood cells (RBC) were incubated for 1hr at room temperature with dilutions of lectins from Arachis hypogaea (100 μg/ml) or Lotus tetragonolobus (100μg/ml) in PBS [pH 5.5]; and the effects on haemagglutination noted. Table 5.2 shows that Lotus tetragonolobus lectin inhibited viral haemagglutination. This lectin is specific for fucose attached via a α 1 - 6 linkage to N - acetylglucosamine on N - linked glycoproteins, and suggests that this moiety may play a part in virus binding to the red cell membrane. Arachis hypogaea lectin however, failed to inhibit haemagglutination, suggesting that the complex sugar residue, Gal β1 - 3 GalNAc (preferentially to α Gal) which it recognizes, does not represent an important site for virus binding. This latter result is unexpected since it disagrees with the findings of Seganti et al. (1987) who proposed a role for galactose residues in virus bindings.

To follow up these results, we attempted to inhibit haemagglutination with porcine thyroglobulin (a large glycoprotein known to be fucosylated), and fucose, in an attempt to compete with the cell receptor for HAV binding. In addition, haemagglutination inhibition was also attempted with foetal calf serum, since Zajac et al (1991) suggested that FCS interfered with virus binding to susceptible cell lines; and heparin to see if HAV shares a cell binding site with herpes simplex virus. Thus, HAV was incubated with dilutions of porcine thyroglobulin (100μg/ml - 10pg/ml), L - fucose (100μg/ml - 10pg/ml), foetal calf serum (10% - 0.00064%) and heparin (100μg - 10pg/ml) each in PBS [pH 5.5] prior to a haemagglutination assay. Table 5.3 goes some way to confirming the hypothesis that fucose is involved in binding.

At a concentration of 1μg/ml or greater, the porcine thyroglobulin has saturated the cell binding sites on the virus, abolishing binding of virus to the RBC. L-fucose does not inhibit haemagglutination and this indicates that the

Lotus tetragonolobus	100mg/ml -
Arachis hypogaea	100mg/ml +

Table 5.2 Inhibition of HAV - induced haemagglutination by lectins. (- denotes no haemagglutination, + denotes haemagglutination).

Foetal calf serum (%)	10 -	2 -	$4x10^{-1}$ -	$8x10^{-2}$ +	$1.6x10^{-3}$ +	$3.2x10^{-4}$ +
Porcine thyroglobulin (mg/ml)	100 -	10 -	1 -	$1x10^{-1}$ +	$1x10^{-2}$ +	$1x10^{-3}$ +
L - fucose (mg/ml)	+	+	+	+	+	+
Heparin (mg/ml)	+	+	+	+	+	+

Table 5.3 Inhibition of haemagglutination by various agents. (- denotes no haemagglutination, + denotes haemagglutination).

position of the fucose molecule within the glycoprotein may also be important. As would be predicted from the results of Zajac et al, (1991) FCS inhibited haemagglutination down to a concentration of 0.4%. The failure of heparin to inhibit haemagglutination indicates that heparan sulphate does not compete with the cell for binding to the virus, and suggests that this cell surface moiety does not play a part in virus binding.

5.4 INHIBITION OF VIRUS BINDING TO CELLS WITH LECTINS

Having determined the effects of lectins on HAV - induced haemagglutination, it was necessary to investigate the effects of the same conditions on virus binding to FRhK-4 cells. This was primarily undertaken to discover whether or not red blood cells represent an adequate model for the binding of HAV to FRhK-4 cell monolayers.

Confluent monolayers of FRhK-4 cells in a 24 - well plate were gently washed twice in PBS and incubated for 1 hr at room temperature, with the following lectin solutions; Arachis hypogaea (100 μg/ml), Lotus tetragonolobus (100μg/ml), Triticum vulgare (100μg/ml), or Lens culinaris (100μg/ml), and then gently washed three times in PBS. 8000 cpm [^{125}I]-HAV in binding buffer [1.8 mM Ca^{2+}] was added to each of the wells. All wells were then left for 2 hr at 4° C. The labelled virus was then removed, but retained in 4 ml stoppered plastic test - tubes, as were the subsequent three PBS washes. The cells were solubilized in 1M NaOH, and placed in plastic test tubes. The results were then counted using an LKB gamma counter. Table 5.4 shows the effects of lectins on virus binding. It appears that the results obtained for the haemagglutination inhibition by lectins cannot be reflected in binding inhibition. This may mean that the inhibition of haemagglutination by fucose - specific lectins is not as a result of its specificity for fucose, but by general steric hindrance. The alternative is that the specificity for fucose is relevant, but that the receptor on

Lectin	% Binding
Control	100 ± 9
Lens culinaris	149 ± 21
Triticum vulgare	82 ± 13
Limulus	142 ± 9
Arachis hypogaea	100 ± 13
Lotus tetragonolobus	79 ±28

Fig. 5.4 Inhibition of ^{125}I-HAV binding to FRhK-4 cells by pre-incubation of the cell surface with lectins. 8000 cpm of ^{125}I-HAV was incubated with 100 m g/ml of various lectins for 2 hr at 35°C. The virus was then incubated for 2hr at 4°C with a monolayer of FRhK-4 cells. The degree of binding was then established as has been described. Maximal binding (control 100%) 85cpm.

red blood cells is able to bind HAV whilst being different from the receptor on susceptible cell lines such as FRhK-4.

6. DISCUSSION

6.1. HAV BINDING STUDIES

Temperature Dependence

To optimize the binding of HAV to FRhK-4 cells, binding was compared between two temperatures, 4°C and 35°C. This was undertaken because all binding experiments were to be performed on unfixed live cells, to more accurately reflect *in vivo* conditions. The complication with this approach is that at physiological temperatures virus is rapidly internalized, leading to complex kinetics and difficulties with interpretation of data. To overcome this it would be more convenient to perform assays at 4°C, where membrane fluidity and virus internalization is greatly reduced. This would allow equilibrium between virus and receptor to be established.

As well as simplifying conditions, it has also been shown that other viruses apparently bind better at lower temperatures (Joklik and Darnell, 1961; Fenwick and Cooper, 1962 and Crowell and Philipson, 1971) resulting in a more sensitive assay. This may be due to the fact that at 35°C membrane receptor proteins are not as evenly dispersed as they are at 4°C. Consequently there are less available binding sites on the cell surface, or the ability of the virus to bind has been reduced. For all of these reasons it was important to perform the investigation shown in section 3.1.

The results shown in Fig.3.1 show that more virus is bound at 4°C after 2 hr than at 35°C, a result later confirmed by Zajac et al, (1991). This would indicate that either receptor capping has taken place to such an extent that all available binding sites are saturated after a 2 hour incubation at 35°C, that virus is being internalized and viral proteins recycled or that the interaction of virus and receptor is more stable at 4°C than 37°C. To completely remove the possibility of receptor capping, cells could be fixed with 80% acetone to halt

internalization, prior to saturating the monolayer with [^{125}I]-HAV and comparing the resulting B_{max} with that obtained from the saturation curve obtained with [^{125}I]-HAV binding to unfixed cells at 4°C.

The effect that an increase in temperature apparently has on binding may add to the many possible explanations as to why HAV replicates so poorly in tissue culture. The accepted method for the infection of a monolayer with HAV is to incubate the monolayer with virus in PBS for 2 hr at 35°C. This, in light of the results obtained in section 3.1, may not be the most efficient way. One route of investigation that may yet be explored is to compare yields of HAV from monolayers infected at 4°C and 35°C. If these results reflect the *in vivo* condition, it would be expected that a lower m.o.i. is required at 4°C to obtained the same yield. These results should agree in principle with the B_{max} estimation as described above.

As a consequence of the obtained results all subsequent binding experiments were undertaken at 4°C.

Cellular Tropism

The host range of HAV is generally regarded as very limited, being confined to cell lines of primate origin. Information regarding the cell types the virus infects (e.g. epithelial, fibroblastic) was, and still is, less clearly defined, with reports of virus antigen recovered from kidney, epithelial, lymph node and hepatocyte tissue (Fagan et al, 1990) as well as cultured cells which are not generally permissive to HAV (Day, personal communication). There were however, no data concerning the virus's binding tropism, though initially we assumed that binding tropism would be limited to cells that the virus is known to infect. It was therefore considered important to begin to investigate the binding range of the virus to try and see if this assumption was correct.

The results obtained in section 3.2 suggest that our assumption was not correct, with similar binding curves obtained with FRhK-4, CHO and WRC cells as well as the agglutination of erythrocytes by various preparations of HAV. This

would at first indicate that the possession or absence of a specific receptor by a cell does not dictate which cells the virus infects.

Unfortunately, as is often the case with HAV (as a result of its tendency to form non-specific interactions), it has yet to be established whether or not binding of HAV to CHO, WRC and red blood cells is of a specific nature. This question is of considerable importance, as its answer would indicate whether or not the initial cellular receptor - virus interaction could still mediate cell and tissue tropism. This data could be obtained by successfully saturating a monolayer of CHO or WRC cells, or blocking binding with the use of HAV - specific neutralizing monoclonal antibodies. In addition, Anderson et al (Meeting abstract) have recently copurified a putative cellular receptor for HAV with bound virus, and are in the process of isolating it. Once sufficient amounts have been purified then some sequence analysis can take place. This sequence information could then be used for, amongst other things, the full determination of the distribution of the cellular receptor in different cell types, by looking for receptor - specific mRNA.

If cellular tropism is not determined by binding to specific membrane proteins, then it must be determined at an intracellular level, as with several other virus families. Influenza virus for instance, utilizes intracellular proteases to cleave and mature haemagglutinin for use during subsequent cycles of replication. The obvious step, if it were to be shown that the HAV host range is determined by intracellular mechanisms would be to undertake work to detect the presence of intermediates throughout its replicative pathway. This would have to begin with the investigation of whether virus is internalized in the first place, and may involve more in depth studies at the cell surface. Herpes Simplex Virus for example, bears several membrane glycoproteins but the resulting interaction of the virus with its target cells is not a simple 'binding - internalization' sequence of events. Instead, one of the virus's glycoproteins has been implicated in penetration as a separate event from binding (Johnson et

al, 1990). This could still be the case with HAV. The myristylation status of HAV, and its comparison with other picornaviruses (as described in 1.1.6) may also have an important bearing on internalization, although the mechanisms of picornavirus penetration of cellular membranes has yet to be fully described.

The saturation binding curves described in chapter 3, in conjunction with the recent isolation of the cellular receptor, have also shed light upon the interaction of HAV with permissive cell lines. The linear - linear plot and the semi - log plot of saturation are sigmoid, an indication of positive cooperativity. This observation is reinforced by the close fit of the data to a curve described by the Hill equation, and the inability to fit the data to a linear plot for classical Michaelis - Menten - like binding, namely the Scatchard plot. Furthermore, the Hill coefficient also suggests that allosteric mechanisms are taking place.

A more reliable picture of the saturation of FRhK-4 cells with [^{125}I]-HAV would have been established if more data points had been obtained. Unfortunately the quantity of virus required to completely saturated the monolayer and obtain a non-specific binding curve were beyond the capacity of the tissue culture facilities available.

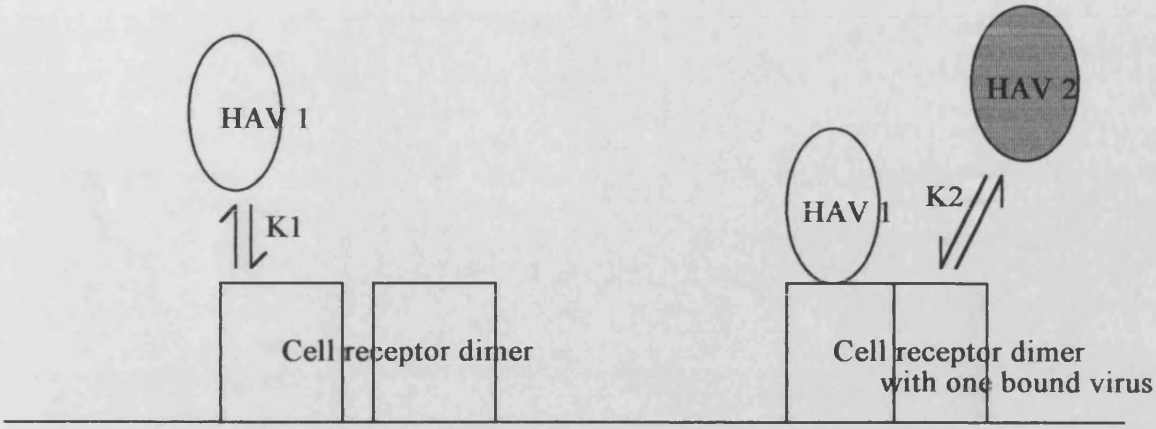
Though, initially the mechanisms of an allosteric interaction are hard to envisage, the evidence of Anderson et al (Meeting abstract) that the cellular receptor is a homodimer which also binds HAV in its monomeric form, lends weight to the suggestion that binding may not be of a simple nature, as it is not uncommon that the binding of a ligand to a monomer of a dimeric receptor induces a conformational change in the adjacent monomer. There is however another possible explanation; HAV has a tendency to aggregate, indicating that HAV has an affinity for itself. If one virion has bound to its receptor, following Michaelis - Menton kinetics, it is quite possible that its presence increases the affinity of the dimer - virus complex for a second virion, leading

to apparent positive cooperativity, provided the affinity of virion for virion is greater than that of virion for receptor (see Fig 6.1)

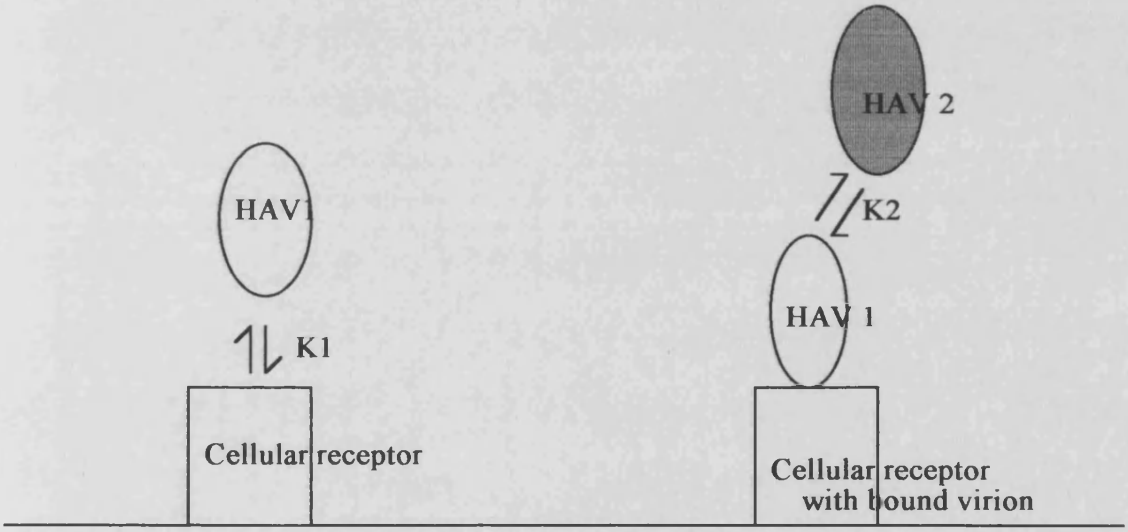
This hypothesis is reinforced by the observation that viruses often exhibit changes in structure upon binding to cells, revealing more hydrophobic structures (Paul et al., 1987; Chow et al., 1987) which in turn would mean that a bound virus is even more likely to attract an unbound virus than a free virus would. If indeed this increased affinity of free virus was dependent upon a structural change in the bound virus, then this interaction would also be saturable, as is demonstrated by the results obtained. Unfortunately it is not easy to see how this scenario might be disproved, without more information about the structure of the receptor.

There are also some artefactual explanations for the shape of the Scatchard and semi-log plots. One problem with the Scatchard plot is that if the original preparation of ligand is not pure, and the impurities become radiolabelled, then an excess of non-binding impurities can lead to this type of plot. This was overcome by ensuring that the preparations of virus were very highly purified, with SDS-PAGE analysis of the preparations routinely undertaken; typical results are shown in fig. 6.2 and fig. 6.3. Additionally the non-specific binding element of a saturation curve may influence its shape and give misleading results. Although a complete estimation of HAV - non-specific binding was impossible due to the constraints of virus availability, (a monolayer must be saturated with cold virus before establishing the extent of non-specific binding with [^{125}I] - HAV) the influence of a linear non-specific curve on the saturation curve obtained could be observed on a purely hypothetical basis. When this was done the resulting "specific curve" remained sigmoidal, suggesting that the original result obtained was real.

The number of receptor copies on FRhK-4 cells can be estimated from the extrapolated saturation curve, and indicates that there are approximately 1500 receptors / FRhK-4 cell.



A



B

Fig 6.1 Two diagrammatic explanations for the apparent positive cooperativity. Example A shows a classic allosteric interaction with the first virion binding to a dimeric receptor and, as a result increasing the affinity of the receptor for a second virion (K2 > K1). Example B shows a second virion binding to the cell receptor via a first virion rather than directly to the cell receptor. This results in positive cooperativity if HAV has a higher affinity for itself than for its cellular receptor (K2 > K1).

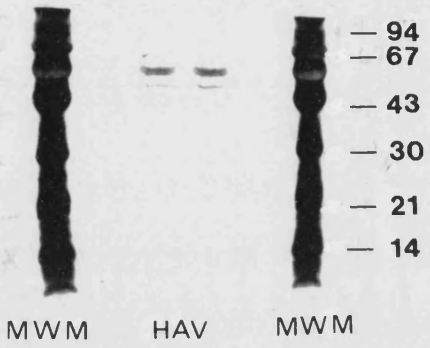


Fig. 6.2 SDS-PAGE analysis of a typical purification of HAV showing capsid proteins VP1, VP2 and VP3.

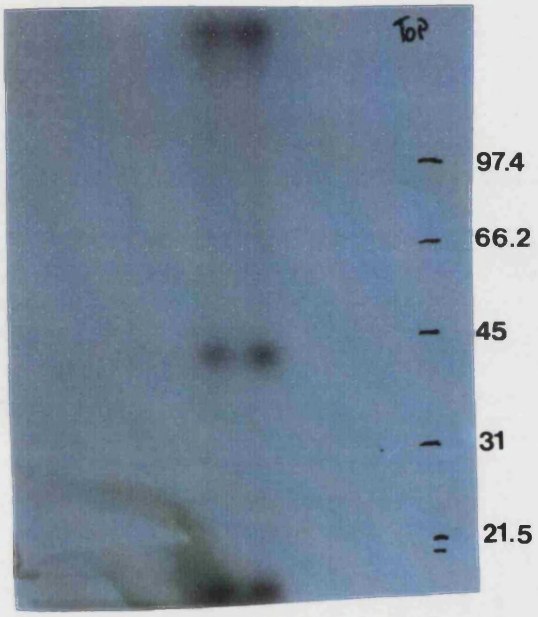


Fig. 6.3 SDS-PAGE analysis of [^{125}I] - HAV.

Iodination $\approx 2.2 \times 10^6$ cpm /1μg of HAV protein

$\Rightarrow B_{max} = 5031$ cpm / 1.25×10^5 cells

$\Rightarrow B_{max} = 2.3 \times 10^{-3}$ μg of HAV protein / 1.25×10^5 cells

$\Rightarrow B_{max} = 1.75 \times 10^8$ virions / 1.25×10^5 cells

$\Rightarrow$ **B_{max} = 1380 virions / cell**

This compares with a range of 10^3 to 2.5×10^4 for Foot-and-Mouth disease virus (Baxt and Morgan, 1986), 3×10^3 to 15×10^3 for rabies virus (Wunner and Reagan, 1986), 6×10^3 to 6×10^5 for coxsackievirus B3 (depending on cell type) (Hsu et al, 1990) and 3×10^3 for Poliovirus (Mendelsohn et al, 1989). An estimation of the K_d for the interaction can also be given from the $S_{0.5}$ calculated by the FigP curve fitting program.

$S_{0.5}$ = 47716 cpm {iodination = 2.2×10^6 cpm /1ug virus }

= 2.17×10^{-2} μg virus protein

= 1.63×10^9 virions

= 2.72×10^{-9}μmoles in 200μl

= 1.36×10^{-5} μM

Virus receptor complex formation

The method virus - receptor complex production for the purification of virus cellular receptors has been shown to be successful when the raising of an anti - receptor monoclonal antibody proves to be impractical, as with Coxsackievirus B3 (Mapoles et al, 1985).

Fig 3.7 illustrates a density shift for [^{125}I] - HAV having been incubated with solubilized FRhK-4 cell membranes. Whether or not the protein associating with the virus represents a cellular receptor for HAV remains conjecture, particularly since the virus - receptor complex recently isolated by Anderson et al (Meeting abstract) does not progress as far down a sucrose density gradient as the putative virus - receptor complex shown in fig. 3.9 (A) (which may represent a virus receptor complex but as part of a large virus aggregate) Fig. 3.9 however, also shows a 'shoulder' on the major virus peak (B) which would

be in approximate agreement with the rate of sedimentation of the virus - receptor complex isolated by Anderson et al (Meeting abstract). To resolve this question it would have been useful to repeat the experiment, varying the run length and range of sucrose gradient, to see if peak B could be further resolved. Having resolved a peak, then the virus - receptor complex could be reiodinated, and the molecular mass of the receptor established by subjecting it to SDS-PAGE. Again, it is important to point out that peak A was obtained several times, in efforts to improve the yield of this peak. (Figs. 3.7 - 3.9). Attempts to identify the constituents of peak A yielded no extra information. There was insufficient counts of bound virus to detect it by SDS-PAGE. Subsequent iodinations of the whole of peak A yielded nothing. This was probably due to unavoidable losses during the procedure, such as non-specific binding to the size exclusion column (even though these columns were preblocked with albumen). It is clear that yields must be improved before further investigation can take place.

Surface labelling (or *in vitro* labelling) of the target cells may improve quantities of purified virus - receptor complex isolated since this would be less likely to damage the virus and therefore the virus - receptor interaction.

The next step would be to identify this virus - receptor complex by sandwich ELISA (see 2.11) rather than relying on virus iodination. As a result, the purified complex could be used as an immunogen for the production of an anti-receptor antibody, reducing the number of clones which would have to be tested.

These preliminary experiments suggest that this method might be useful for receptor isolation. The obvious limitations of the method are that purified virus is required, and only small quantities of cellular receptor can be isolated at a time.

6.2. HEPATITIS A VIRUS - SPECIFIC MONOCLONAL ANTIBODIES.

The neutralization of viruses by monoclonal antibodies and convalescent sera can, in many instances, be extremely complex, with the inhibition of binding being one of the more uncommon mechanisms (Dimmock, 1984).

It is thought that Influenza Virus, for instance, is neutralized by the inhibition of transcription. A neutralizing monoclonal antibody may initially bind to the virus's envelope glycoprotein, but the neutralized virus is still able to bind to the target cell, be internalized and uncoat. The viral transcriptase complex has however been altered, and no RNA or viral proteins can be detected. The disabling of the viral transcriptase complex has been shown to occur at the point when the antibody binds to the virus haemagglutinin (HA) and remains on the cell surface once the virus has been internalized, playing no further part in neutralization. The mechanism by which the neutralization is transduced from the virus envelope to the transcription complex is not understood (Dimmock, 1984).

Viruses may also be neutralized by more than one mechanism. Poliovirus for example, is probably neutralized by the inhibition of binding, but also by mechanisms by which the structure is altered such that the virus pI remains at 4, which is not compatible with infection of the host cell (poliovirus varies between a pI of 4 and 7, the latter form being infectious).

The results obtained for HAV suggest that this virus is neutralized by the binding of neutralizing antibodies to the area of the virus capsid which interacts with the target cell, since the ability of antibody to neutralize the virus correlates with its ability to inhibit binding to red blood cells and FRhK-4 cells. If this hypothesis proves to be accurate, it would fit the observation that there is only one naturally occurring HAV serotype, since to escape a neutralizing polyclonal serum would almost certainly prove fatal. As a consequence, this would mean that the 'canyon hypothesis' which has been applied to other

picornaviruses, does not apply to HAV, since to neutralize by this route would clearly need an exposed binding site. There are reports of HAV escape mutants which are still viable (Ping and Lemon, 1992), but their ability to bind to cells and replicate as efficiently as wild type virus has not been fully investigated, so it seems possible that these viruses are not able to compete with wild type virus in a mixed population, even if they do continue to exist in a single serotype environment.

Unfortunately, the failure as yet to fully correlate the haemagglutination result with the inhibition of [^{125}I]-HAV binding to FRhK-4 cells means that the hypothesis has yet to be confirmed.

The most likely reason why 14H should partially inhibit [^{125}I]-HAV binding to FRhK-4 cells but not RBC is that the receptor on non-permissive cells (such as red blood cells) and permissive cells (such as FRhK-4 cells) are not the same, or are in a different immediate environments, and that cell tropism is still determined at the cell membrane, as has been discussed in chapter 6.1.

A situation could be envisaged where the epitope of the non-neutralizing antibody, 14H, is close to the neutralization site on the virus capsid and is effective in inhibiting binding of virus to FRhK-4 cells but not red blood cells. This probably means that the receptor on red blood cells is more exposed than on FRhK-4 cells.

It is also possible that there are two different active binding sites on the virus which would allow the virus to bind to different target cells at different binding sites. The different optimum [Ca^{++}] and pH may then be explained by the two different virus - target cell interactions. It would also have interesting ramifications for the pathological mechanisms of the virus since the pH where enteroviruses first interact with the host (the intestines) is generally more acidic than that of the intercellular space surrounding hepatocytes.

It may be difficult however, to reconcile this hypothesis with the results obtained with MAbs 813 and 10.09, which interfered with the binding of HAV

to FRhK-4 cells and red blood cells suggesting that these monoclonal antibodies bind to a portion of the virus which interacts with both FRhK-4 and red blood cells.

It would interesting to construct HAV saturation binding curves for RBC as this would indicate what kind of interaction exists between HAV and RBC. It would then allow comparisons with the FRhK-4 cell - HAV interaction.

It must be pointed out that the quantitation of the monoclonal antibodies described in section 2.9 suffers at the hands of limited information regarding the affinities of the mAbs 813, 10.09 and 14H for HAV. Though the assumption is that the degree of competition binding relates to the site on the virus where the antibodies are binding, (14H competes with [^{125}I]-10.09 less than 813 because it is binding to a different area of the virus capsid), it may also indicate different affinities of the antibodies for the same location.

This could be overcome by gaining some information about the affinities of 813, 10.09 and 14H for HAV or by repeating the exercise using [^{125}I]-14H and [^{125}I]-813 as the competed antibody to see if the same conclusions can be drawn.

In addition the quantitation of the mAbs by direct ELISA suffers from the doubt as to how much of the total IgG present in the ascitic fluid preparations is directed against HAV and how much is normal murine production. Though this could be countered by quantitating the antibody using the sandwich ELISA the error is unlikely to be greater than 10% since in general around 90% of ascitic fluid IgG is directed against the target antigen. The different monoclonal antibody isotypes may also influence the result since the affinity of the detecting antibody for different mAbs may vary.

6.3. INHIBITION OF BINDING

The limited inhibition studies described in Chapter 5 give some insight into the structure of the receptor. The enzyme pretreatment of the cell surface prior to binding assays indicates that the receptor is comprised fundamentally of protein. This is not surprising as the majority of receptors are proteins. There are however, exceptions. Influenza virus, as an example, utilizes sialic acid as a primary binding site, and though this moiety is often associated with proteins, this is not essential. As a result the trypsin pretreatment of a cell will not entirely abolish influenza virus binding. The neuraminidase result obtained for HAV, at first appears to suggest that sialic acid does not play a part in virus binding (HAV binding remained at 86% after neuraminidase treatment of the cell). However, the degree of removal of sialic acid was not established. Endoglycosidase F treatment of the cell surface was investigated to see if removal of N - linked carbohydrate residues would effect binding, and thus to see if the HAV receptor was indeed a glycoprotein. Initially it would appear that this is not the case, but the ability of this enzyme to cleave effectively when a protein is folded is doubtful. This result therefore must be viewed with considerable caution.

The degree of enzyme digestion of the cell surface could be tested in two ways. Viruses or other ligands known to bind to certain moieties could be used in binding assays. Influenza virus for instance could be utilized to quantify the degree of removal of sugars from the cell surface by endoglycosidase F and neuraminidase whilst Rhinovirus or Poliovirus could give an indication of protein digestion by trypsin.

Assaying for peptides and reduced sugars in the cell supernatant would also allow quantitation of the degree of degradation.

Given sufficient [^{125}I]-HAV a time course of binding against time of enzyme treatment would also confirm the results obtained.

The haemagglutination inhibition studies, though never intended to represent a definitive study of the possible components of the cellular receptor, are a relatively simple screening assay to determine whether certain compounds affected binding of virus to red blood cells. The lectin studies at first sight appear to lend evidence to the argument that sugar residues may play an important role in binding, and more specifically, suggest that the less common fucosylation of oligosaccharides may be particularly relevant. The subsequent use of thyroglobulin (a glycoprotein known to bear fucosylated oligosaccharides), and free L - fucose, to inhibit haemagglutination was an attempt to confirm these results. The results of these experiments did fit the hypothesis, with low concentrations of thyroglobulin inhibiting haemagglutination. The inability of fucose to affect binding was not surprising, considering its small size. These results could be complemented with the use of more specific enzymes to specifically remove fucose, such as fucosidase. The well documented effect of foetal calf serum on binding (again confirmed here) also reinforced this conjecture, since foetal calf serum contains several glycoproteins bearing fucose. However, when confirmation of these results was sought after in the more applied environment of the binding assay, the Lotus lectin (specific for fucose) failed to inhibit binding as conclusively as in the haemagglutination assay . Due to lack of available virus and the constraints of time, it was not possible to perform a dilution series to indicate whether or not the virus was saturated by the lectin solutions which were incubated with the virus.

There remains the possibility that the receptor for HAV on non-susceptible cell lines (such as red blood cells) and permissive cells (such as FRhK-4 cells) are not the same, or are in different immediate environments. One difference may be that fucose only plays a part in binding to red blood cells and may have a bearing on why HAV binds but does not infect certain cell lines. Table 6.4

	Red Blood Cells	FRhK-4
pH	5.5	7.4
[Ca^{++}]	Not needed	Essential
Non-neutralizing antibody, 14H	No effect	Some effect
Fucose-specific lectin	Inhibits HA	No effect

Table 6.4 Summary of differences between interaction of HAV and red blood cells and FRhK-4 cells.

summarizes this and other notable differences between binding of HAV to FRhK-4 and red blood cells.

Again it is worth noting the possibility of two separate binding sites on the virus capsid which may interact with two different sites on FRhK-4 and red blood cells (as has been mentioned previously). This scenario, of course would probably give very different results in a study of this kind because different moieties on the different cell surface would be involved in binding the virus.

It would also be useful to look at the effects of these conditions on the binding of HAV to other susceptible cell lines such as CHO and WRC cells to see if any correlation arises. Equally important would be to repeat the saturation and temperature analysis with non-susceptible cell lines to identify any other differences such as receptor affinity. It may then be possible to draw some conclusions as to why HAV binds to certain cell lines but does not (apparently) infect them.

The prevailing problem that existed with binding studies with HAV, is the virus's well documented susceptibility to aggregation and non - specific interactions with proteins. As a result, the haemagglutination inhibition studies must be taken as a preliminary investigation, but must also be backed up with more in depth work involving binding to susceptible cell lines. It must also be borne in mind that lectins are large polypeptides, and their effect on binding may come as a result of general steric hindrance of the binding site, rather than specific interference with certain sugar residues.

6.4 CONCLUSIONS

The production of usable quantities of HAV represented a problem throughout the project. A problem that hampers most workers in the field of HAV research. Attempts to improve the yields of virus included switching briefly to BS-C-1 cells as the host, an approach favoured by many workers who claim

that this cell line gives better cytopathic effect with the HM175 strain of HAV. In our hands however there was no improvement in virus yields and indeed HAV - mediated plaques were first experienced with the FRhK-4 cell line. Attempts to move production into roller bottles with the resulting vast increase in cell monolayer area also afforded nothing as the cells did not grow efficiently. Indeed the behaviour of FRhK-4 cells in roller bottles was so unexpected that mycoplasma testing via the Hoechst stain was instituted to try an explain it. This gave negative results whenever used.

It is a running theme throughout the project, particularly with the saturation curve analysis that an increased availability of HAV antigen would have improved the quality of results obtained.

However, certain definite conclusions can still be made. The receptor is certainly a protein. This has been shown both by the interference of binding by trypsin treatment of the target cell, and by the staining of the non - viral portion of the virus receptor complex isolated by Anderson et al, (Meeting abstract). The role of protein associated moieties unfortunately remains extremely vague. This however can be probed, given time and sufficient virus, using the methods outlined above, namely inhibition of haemagglutination by any number of compounds and lectins as a general screen, followed by application of the same methods for virus binding to FRhK-4 or BS-C-1 cells. Enzyme treatment of red blood cells, FRhK-4 and BS-C-1 cells would also provide valuable information.

The saturation of a monolayer of FRhK-4 cells with virus indicates that the cell surface can be saturated, that the receptor copy number is not high, and that mechanisms of positive cooperativity are taking place as a result of an allosteric interaction at multiple sites on the receptor. This finding appears to support the findings of Anderson et al (Meetings abstract) that the receptor may exist as a dimer. The binding tropism of the virus has yet to be confirmed, inasmuch as a specific interaction of virus with cell lines unable to support its replication

has not been shown. Again, resolution of this question does not present any complicated technical problems. Saturation of a monolayer of such a cell line would be sufficient to show the interaction to be specific. If this proved to be the case, far more work would be required to discover what determines which cell types the virus will infect.

Although the non-neutralizing antibody reduced HAV binding to around 10%, the results obtained with the neutralizing antibodies strongly suggest that the virus is neutralized at its cell binding site.

It is still important to try and produce a cell receptor monoclonal antibody, for at least two reasons. Firstly, a monoclonal antibody would allow a large scale purification of the receptor and subsequent in depth investigation of its structure. Secondly, although eventually the binding tropism of the virus in tissue culture will be described, it may not be possible to relate the results to an *in vivo* situation. A monoclonal antibody to the receptor, conjugated to an appropriate label, could be used in immunocytochemical analysis of tissue samples to identify which human cells the virus may infect; assuming, of course that there is a correlation between the possession of a specific cell surface receptor and the infection of that cell.

In short, there is still a great deal of work to be done on the infection of cells by HAV, but with the progress that has been made in this area of research it seems certain that many of the outstanding questions will soon be answered.

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